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Mitochondrial Reactive Oxygen Species Production in Excitable Cells: Modulators of Mitochondrial and Cell Function

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Abstract

The mitochondrion is a major source of reactive oxygen species (ROS). Superoxide ($O_2^{\bullet -}$) is generated under specific bioenergetic conditions at several sites within the electron-transport system; most is converted to H_2O_2 inside and outside the mitochondrial matrix by superoxide dismutases. H_2O_2 is a major chemical messenger that, in low amounts and with its products, physiologically modulates cell function. The redox state and ROS scavengers largely control the emission (generation scavenging) of $O_2^{\bullet -}$. Cell ischemia, hypoxia, or toxins can result in excess $O_2^{\bullet -}$ production when the redox state is altered and the ROS scavenger systems are overwhelmed. Too much H_2O_2 can combine with Fe^{2+} complexes to form reactive ferryl species (*e.g.*, $Fe(IV)=O^{\bullet}$). In the presence of nitric oxide ($NO^{\bullet}$), $O_2^{\bullet -}$ forms the reactant peroxynitrite ($ONOO^{-}$), and ONOOH-induced nitrosylation of proteins, DNA, and lipids can modify their structure and function. An initial increase in ROS can cause an even greater increase in ROS and allow excess mitochondrial Ca^{2+} entry, both of which are factors that induce cell apoptosis and necrosis. Approaches to reduce excess $O_2^{\bullet -}$ emission include selectively boosting the antioxidant capacity, uncoupling of oxidative phosphorylation to reduce generation of $O_2^{\bullet -}$ by inducing proton leak, and reversibly inhibiting electron transport. Mitochondrial cation channels and exchangers function to maintain matrix homeostasis and likely play a role in modulating mitochondrial function, in part by regulating $O_2^{\bullet -}$ generation. Cell-signaling pathways induced physiologically by ROS include effects on thiol groups and disulfide linkages to modify post-translationally protein structure to activate/inactivate specific kinase/phosphatase pathways. Hypoxia-inducible factors that stimulate a cascade of gene transcription may be mediated physiologically by ROS. Our knowledge of the role played by ROS and their scavenging systems in modulation of cell function and cell death has grown exponentially over the past few years, but we are still limited in how to apply this knowledge to develop its full therapeutic potential.

I. Introduction

The turn of the century has seen a resurgence of interest in mitochondrial generation of reactive oxygen species (ROS), first discovered and understood during the late 1960s and early 1970s. The renewed interest derives from the newer concept that ROS are not always deleterious to cell function and from the use of more sensitive and accurate techniques and probes to understand the mechanism of ROS generation and scavenging systems. A number of commentaries and well-researched reviews have been published since 2000 on the

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putative mechanisms of ROS production and the complex pathophysiologic effects that ROS exert on cell function; many of these are referenced throughout this review (8, 27, 31, 48, 55, 59, 66, 72, 75, 80, 81, 113, 122, 126, 132, 133, 140, 146, 163, 179, 183, 189, 202, 209, 234, 241, 243, 244, 246, 299, 303, 307, 328). Many of these reviews address very specific aspects of mitochondrial bioenergetics or deal with nonmitochondrial ROS production or both. For this review, we hope to present a comprehensive understanding of mitochondrial ROS production sites and their regulation, ROS scavenging, and physiologic as well as pathologic effects of ROS, particularly as triggers of cell protection.

A. Focus of review

This review focuses primarily on the ROS generated by the mitochondrial respiratory complexes, the sites and conditions for ROS generation, cell regulation of ROS generation and ROS scavenging, cell-damaging effects of ROS *vs.* physiologic regulation of mitochondrial and cell function by ROS, and the emerging role of pharmacologic approaches to manipulate generation and scavenging of specific ROS. Other potential sources of ROS exist in mitochondria (*e.g.*, glycerol-3-phosphate dehydrogenase, acyl-CoA dehydrogenase), but their physiological significance remains to be elucidated (8). This report concentrates on studies of mitochondria in excitable cells with high metabolic rates (*i.e.*, mammalian myocardial and nervous system cells). Mitochondrial density is very high in muscle and nerve cells because of their higher O_2 consumption rate compared with that of most other cell types. The number, size, shape, interconnections, and location of mitochondria are also different depending on cell types (91). Mitochondria also serve different functions within the same cell [*e.g.*, in nerve cells (91) and between the interfibrillary and subsarcolemmal mitochondria in cardiac cells (213)].

This review summarizes studies on how and why ROS are generated by mitochondrial electron-transport complexes in physiologically low amounts, perhaps up to 1% or 2% of the total rate of O_2 consumption (77), to modulate mitochondrial and cell function. A number of topics are addressed: How can ROS be generated when the level of the electron acceptor O_2 is low? What is the source and

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location of ''good'' *versus* ''bad'' ROS? Which are the physiologically relevant ROS, and what are their targets? How are the levels of physiologic ROS modulated? How can generation or scavenging of ROS be manipulated to support cell function when the cell is stressed? Are low O_2–induced cell signaling pathways initiated at the mitochondrial level, and does an increase or decrease in ROS mediate this activity?

This review does not specifically address the significant extramatrix and nonmitochondrial sources of ROS [*e.g.*, cytochrome b_5 reductase, perioxisomes, catecholamines, hydroquinones, plasma membrane oxidases such as NADPH oxidase, lipoxygenases, monoamine oxidases, xanthine/xanthine oxidase, coupled or uncoupled nitric oxide synthase, and eicosanoids pathways, among others (303)]. Most of these compounds can initially lose an electron to form the superoxide anion radical ($O_2^{\bullet -}$) by autooxidation. The nonmitochondrial ROS released or formed in the cytosol are buffered generally under strong reducing conditions by intracellular thiols, particularly glutathione (GSH) and thioredoxin ($TRXSH_2$) by the activities of their reductases (303); this topic is addressed only as applied to mitochondriaderived ROS. The free radical $NO^{\bullet}$ can have profound effects on mitochondrial function (*i.e.*, competing with O_2 at mitochondrial respiratory complex IV and reacting with $O_2^{\bullet -}$ to form peroxynitrite). The source of the $NO^{\bullet}$ may be vascular endothelium, nerve terminals, or other cytosolic sources. Controversial evidence that suggests that $NO^{\bullet}$ may be generated by a mitochondrial NO synthase (23, 24, 136, 137), but this review deals primarily with respiratory complex–derived $O_2^{\bullet -}$ and its products.

B. Ambiguities about ROS

Although much is known of the chemistry of ROS (151), much less is known about the specific molecular sites and conditions for electron leak and $O_2^{\bullet -}$ generation along the electron-transport pathway under pathophysiologic conditions (32, 61). Some topics addressed subsequently: How and why do singlet electrons escape during electron transfer? What are the physiologic pathways for $O_2^{\bullet -}$ release? How can $O_2^{\bullet -}$ be formed during state 3 (ADP-stimulated) respiration when the $\Delta\Psi_m$ is less polarized because of the influx of protons? How can more $O_2^{\bullet -}$ be generated when O_2 levels decrease? Are isolated

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mitochondria a proper model for assessing ROS generation? What is the relative amount of $O_2^{\bullet -}$ generated at sites along the respiratory complexes? Does $O_2^{\bullet -}$ or other ROS function as an O_2 sensor to modulate enzyme activity? What distinguishes physiologic from pathologic release of $O_2^{\bullet -}$? It is hoped that this review will furnish at least satisfactory answers to most of these questions.

II. Overview of Mitochondrial Structure and Function

A. Structure

Mitochondria are membrane-enclosed organelles (1- to 10-μm diameter) that generate most of the cell's supply of ATP; they also have a role in cell signaling and differentiation, Ca^{2+} buffering, apoptosis and cell death, as well as control of the cell cycle and cell growth (322). Many of these processes are triggered or mediated by Ca^{2+} or ROS or both. The number of mitochondria in a cell varies widely by organism and tissue type. Many cells have only a single mitochondrion, whereas others can contain several thousand mitochondria (265, 322). Mitochondrial compartments include the outer mitochondrial membrane (OMM), the inner membrane space (IMS), the inner mitochondrial membrane (IMM), and the cristae and matrix (265). The OMM has a protein-to-phospholipid ratio similar to that of the plasma membrane (~1:1 by weight) and contains large numbers of integral proteins called porins that form channels to allow molecules of more than 5,000 Daltons to diffuse freely across the OMM (265). Larger proteins also can enter the mitochondrion if a signaling sequence at their N-terminus binds to large multi-subunit proteins (translocases) that then actively transport them across the OMM. Disruption of the OMM permits proteins in the IMS to leak into the cytosol, leading to cell death (87). Because the OMM is freely permeable to small molecules, the concentrations of small molecules, such as ions and sugars, in the IMS is the same as in the cytosol (265). However, as large proteins must have a specific signaling sequence to be transported across the OMM, the protein composition of the IMS is different from the protein composition of the cytosol. One protein that is localized to the IMS in this way is cytochrome *c* (87).

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The IMM contains proteins with several functions (225, 265): (a) redox reactions of oxidative phosphorylation, (b) ATP synthase/ATPase, (c) transport proteins that regulate metabolites across the matrix, and (d) protein-import machinery. The IMM has a 3:1 protein-to-phospholipid ratio and is rich in the phospholipid cardiolipin, which contains four fatty acids that help to make the IMM impermeable (322). Unlike the OMM, the IMM does not contain porins and is highly impermeable to all molecules, so that all ions and molecules require membrane transporters to enter or exit the matrix.

Mitochondria vary in number and location according to cell type. Liver cells have ~1,000–2,000 mitochondria per cell, making up 20% of the cell volume (2), and the IMM (+cristae) area is about 5 times greater than that of the OMM. In cardiac mitochondria, the cristae area is much larger, and mitochondrial volume can reach 30% of cell volume. Mitochondria often form a complex 3D branching network inside the cell with the cytoskeleton and tight connections to other organelles. The association with the cytoskeleton determines mitochondrial shape and function (265).

B. Function

The membrane potential across the IMM is formed by the action of the respiratory enzymes and the TCA cycle (225, 322). Each pyruvate molecule produced by glycolysis is actively transported across the IMM and into the matrix, where it is oxidized and combined with coenzyme A to form CO_2, acetyl-CoA, and NADH (322). The acetyl-CoA is the primary substrate to enter the TCA cycle. The enzymes of the TCA cycle are located in the mitochondrial matrix, with the exception of succinate dehydrogenase, which is bound to the IMM as part of complex II. The TCA cycle oxidizes the acetyl-CoA to CO_2, and, in the process, produces three molecules of NADH and one molecule of $FADH_2$, which are a source of electrons for transport along the respiratory complexes, and a molecule of GTP (that is readily converted to an ATP) (322). The redox energy from NADH and $FADH_2$ is transferred to O_2 in several steps *via* the electron transport along the respiratory complexes. The individual proteins of complexes I to V

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specifically interact to form defined supramolecular structures, the socalled ''respiratory supercomplexes'' or ''respirasome'' (114).

Reducing equivalents from the cytoplasm can be imported *via* the malate-aspartate shuttle system of antiporter proteins or fed into the electron-transport system (ETS) by using a glycerol phosphate shuttle. NADH dehydrogenase, cytochrome *c* reductase, and cytochrome *c* oxidase perform the transfer, and the incremental release of energy is used to pump protons (H^+) into the IMS (322). As the proton concentration increases in the IMS, a strong electrochemical gradient is established across the IMM. The protons can return to the matrix through the ATP synthase complex, and their potential energy is used to synthesize ATP from ADP and inorganic phosphate (P_i) (322). This process, called chemiosmosis, was first described by Peter Mitchell (230), who was awarded the 1978 Nobel Prize in Chemistry for his work.

III. Mitochondrial Sources and Mechanism of ROS Generation

A. Requirement for charged membrane, electron flux, and O_2

Molecular O_2 (dioxygen) has two unpaired electrons with parallel spin in different antibonding orbitals. $O_2^{\bullet -}$ arises directly from the reduction of O_2 by transfer of a lone electron to its antibonding orbital (151). The electrons are believed to escape from the mitochondrial ETS at discrete sites during the transport of electrons (Figs. 1 and 2A) from NADH oxidoreductase (complex I) to cytochrome *c* oxidase (complex IV), the ultimate electron acceptor by which O_2+4e^- and $4H^+$ is reduced to $2H_2O$. Oxygen, necessary for life in aerobic organisms, is therefore simply a sink for electrons (66).

The standard reduction potentials of electron carriers in the four mitochondrial complexes span the range of redox potentials from NADH at complex I (—0.32 V) to O_2 at complex IV (+0.82 V) (Fig. 2B). The standard reduction potential for the reduction of O_2 to $O_2^{\bullet -}$ is about —0.16V (307), so many components of the respiratory

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complexes in the ETS are thermodynamically capable of irreversibly transferring an electron to O_2. However, the single-electron transfers are tightly controlled and quickly coupled with the second electron transfer (107, 293, 294). The energy released as electrons are transferred along the respiratory chain is conserved into an H^+ gradient directed outward through the IMM. Ejection of protons alkalinizes the matrix to create a transmembrane H^+ electrochemical-potential gradient, the proton motive force ($\Delta\mu H^+$), comprising the membrane potential ($\Delta\Psi_m$, —150 mV) and the smaller pH-gradient potential (ΔpH_m, —30 mV) (201). The energy stored in the $\Delta\mu H^+$ is partially dissipated as H^+ reenters the matrix at complex V (ATP synthase), which rotates a protein rotor to convert ADP and P_i to ATP. This process is reversible.

B. Reactive and nonreactive O_2 species and reactants

The generation of ROS from mitochondria was first reported in the early 1970s (44, 218). ROS are O_2 molecules alone or bound to elements C, H, or N in different states of oxidation or reduction (Table 1) (151). The primordial mitochondrial ROS is the free radical $O_2^{\bullet-}$ (Fig. 2B), which, as noted earlier, has an added unpaired electron. Although $O_2^{\bullet-}$ can reduce Fe^{3+} to Fe^{2+} and react with other ligands to form other radicals, its usual fate is rapid dismutation (K_d=1.6 x 10^9M/s) to hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD) in the mitochondrial matrix (MnSOD) and IMS and cytosol (CuZnSOD). During the dismutation reaction, the $O_2^{\bullet-}$ is first protonated to the hydroperoxyl radical ($HO_2^{\bullet}$) (K_d 10 x 10^7M/s), and two $HO_2^{\bullet}$ react (K_d 9 x 10^5M/s) to form H_2O_2 and H_2O (151). Thus, the spontaneous dismutation rate of $HO_2^{\bullet}$ is ~100 times faster than that of $O_2^{\bullet-}$ and because it is uncharged, $HO_2^{\bullet}$, unlike $O_2^{\bullet-}$, can cross membranes (104). With a pK_a of 4.8 for the $HO_2^{\bullet}/O_2^{\bullet-}$ pair, very little $O_2^{\bullet-}$ exists as the protonated form in the mitochondrial matrix. It follows that dismutation of $O_2^{\bullet-}$ to H_2O_2 occurs faster when the matrix pH is lower (k=5 x 10^5M/s at pH 7.0 and 10^2M/s at pH 11) (151).

Not all ROS are actually free radicals (*e.g.*, H_2O_2 and peroxynitrite, $ONOO^-$) (Fig. 3). When a free radical reacts with a nonradical, the product is another free radical, whereas the interaction of two free radicals produces a nonradical (151). For example, $ONOO^-$

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is a nonradical formed from the interaction of $NO^{\bullet}$ and $O_2^{\bullet -}$. $ONOO^-$ is stable at an alkaline pH and fairly nonreactive, but ONOO- - is readily protonated (cytosol more than matrix) at cellular pH to ONOOH (peroxynitrous acid), which is very cytotoxic, like $HO^{\bullet}$, and causes depletion of –SH groups and other antioxidants, oxidation of lipids, DNA strand breakage, as well as nitration of and deamination of DNA bases, especially guanine (151). H_2O_2 is not a free radical and is not so strong an oxidant as $O_2^{\bullet -}$, and H_2O_2, like H_2O, diffuses more easily through aquaporins than through lipid membranes (37, 38). H_2O_2 is normally maintained at low levels by catalase and glutathione peroxidase, among others, which convert H_2O_2 to H_2O and O_2.

H_2O_2 also can participate in the one-electron reaction with transition metal ions (*e.g.*, Fe^{2+} bound to organic molecules) to generate various intermediate ferryl species (274, 275), such as $Fe(IV)=O^{\bullet}$, a powerful oxidizing agent that can lead to lipid oxidation and DNA damage, as discussed in section VI. The rate constant, *k*, for reactions of Fe^{2+} is very dependent on the ligand attached to the iron (*e.g.*, it is 2 times greater for Fe^{2+}-ADP than for Fe^{2+}-ATP). Ferryl species exist only in a complex and are produced at their site-specific targets in DNA, proteins, and lipids. Other much slower reactants of H_2O_2 produced are Fe(III)+OH-+$HO^{\bullet}$ (Fenton reaction); this reaction is 30 times slower than that of ferryl compounds with H_2O_2, so the hydroxyl radical ($HO^{\bullet}$) is formed in very small amounts and has no specific reactivity with biomolecules (144). $ONOO^-$ is also a stable oxidant that, in its protonated form, ONOOH, can produce oxidative changes in lipids, proteins, carbohydrates, and nucleic acids. Thus, although $O_2^{\bullet -}$ and $NO^{\bullet}$ are the initial free radicals, H_2O_2 rather than $O_2^{\bullet -}$ is mostly regarded as the relevant biologically active metabolite.

C. Assessing ROS generation

The measurement of the various ROS is dependent on suitable techniques to assess levels of ROS. The technique should not directly produce ROS or produce artifacts. Preferably the technique should directly measure intra- *versus* extramatrix generation of $O_2^{\bullet -}$. The most direct and selective approach to measure specific ROS, notably in the matrix, is to use electron paramagnetic resonance (EPR) (16, 129, 151). EPR is based on absorption of microwave radiation stimulated by

an electromagnetic field in molecules such as free radicals and transition metal ions with unpaired electrons. Adsorption of microwave energy creates two distinct energy levels from the unpaired electrons as they transition from a lower state to a higher state. The number of unpaired electrons present in a sample is proportional to the amplitude of the ESR signal (111). Only stable ROS that accumulate to measurable levels can be identified directly at biologic temperatures, so either extreme cooling or spin traps, which involve reaction of the radical with an adduct that is stable, is used. Examples of EPR signals are shown in Fig. 4.

The majority of recent studies on mitochondrial ROS production use either the intramatrix fluorescence indicators 2'7'-dichlorofluorescein (DCF) or the fluorescent Amplex Red/horseradish peroxidase technique to assess H_2O_2 that is released or converted in the buffer (''cytosol'') surrounding the mitochondria or both. Figure 5 shows typical recordings of the rate of accumulation (slope) of H_2O_2 by the latter technique. Because of the high catalytic activities of MnSOD and CuZnSOD, most $O_2^{\bullet -}$ is rapidly converted to H_2O_2, which is readily permeable to the IMM. Therefore, the measurement of intra- *versus* extramatrix-generated $O_2^{\bullet -}$ is indirect and incomplete because of its rapid dismutation to H_2O_2 and the sidedness (intra- or extramatrix) of $O_2^{\bullet -}$ release.

In isolated tissue, other options exist to assess ROS. One is the shift in absorbance with reduction of cytochrome *c* by $O_2^{\bullet -}$; other techniques are based on release of photons by chemiluminescence probes, such as liminol or lucigenin, on reaction with ROS (111). Dihydroethidium (DHE), also known as hydroethidium (HE), is a rather specific fluorescent marker for $O_2^{\bullet -}$. $O_2^{\bullet -}$ nonenzymatically converts DHE to 2-hydroxyethidium (2-OH-E^+) or a precursor (331), which appears to be rapidly made, is labile, and fluoresces at a slightly shorter wavelength (more red) than the heme-peroxidase oxidation product ethidium that can intercalate to bound with DNA. Thus, the 2-OH-E^+ signals fluctuate with the $O_2^{\bullet -}$ generated in the cell, as now shown in many studies by using spectrophotometry (5, 70, 71, 181, 182, 247, 269, 271, 298, 316, 317). Figure 6 displays a typical recording of $O_2^{\bullet -}$ assessed by DHE fluorescence in intact hearts. Note the dynamic effects of changing temperature in the presence and

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absence of drugs. Caution in the use of DHE (and other probes) lies in its oxidation to ethidium by cytochrome *c* in the absence of ROS (33). Because the DHE product is altered by drugs that act on the mitochondrion (71), it is assumed that mitochondria are the major source of $O_2^{\bullet -}$ by this method in isolated tissue. A derivative of DHE is MitoSOX Red, which has a phosphonium group that selectively targets and enters mitochondria in response to the negative membrane potential; thus, imaging of the signal in cells indicates a mitochondrial source of $O_2^{\bullet -}$ (273).

Another method that depends on the interaction of $O_2^{\bullet -}$ and $NO^{\bullet}$ is the formation of dityrosine from tyrosine by $ONOO^-$ (29); Fig. 7 shows an example of the dynamic changes in dityrosine formation in intact hearts during a change in temperature and drug treatment. It is assumed that the induced shift in wavelength, assessed with spectrometry, is an extracellular indicator of $ONOO^-$ (71, 247, 248). Nitration of tyrosine (no color) by $ONOO^-$ to 3-nitrotyrosine (yellow color) is another indicator of reactive nitrogen species usually identified in proteins (151).

D. Sites and conditions for mitochondrial ROS generation

The specific molecular sites of electron leak are not known with certainty, but a great deal of intense investigation has led to sites within complexes I and III (Figs. 1 and 2A). Specific mitochondrial inhibitors (64, 197) (Table 2) are typically used to (a) force electrons to leak outside of the very tight single-electron transfer mechanism (*e.g.*, antimycin A) leading to $O_2^{\bullet -}$, or (b) to block electron leak (*e.g.*, stigmatellin or rotenone) with glutamate as substrate. For example, the increase in ROS induced by antimycin A can be abolished by adding either stigmatellin or myxothiazol (256). In the presence of glutamate, the inhibitors rotenone, antimycin A, and stigmatellin can each increase ROS. However, in the presence of succinate as substrate, antimycin A can increase, whereas both stigmatellin and rotenone can decrease ROS (255, 256). Thus, the possible pathways have been identified primarily with substrates and inhibitors by using deductive reasoning, with the understanding that the mechanism of

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electron leak may be quite different in a more-relevant pathophysiologic situation.

E. Complex I (NADH ubiquinone oxidoreductase)

This transmembrane complex (Fig. 1) oxidizes NADH [reduced from the transfer of electrons and H^+ from tricarboxylic acid (TCA) intermediates], uses coenzyme Q_{10} (ubiquinone, Q) as the electron acceptor and is coupled (as are complexes III and IV) with proton pumping, thus contributing to the proton motive force, $\Delta\mu H^+$. The actual mechanism of proton pumping is almost completely unknown, in part to the large size of complex I and the difficulty in measuring the intermediates in the coupling reaction (201). Complex I is one of two major sites of entry for reducing equivalents; the other is complex II, succinate dehydrogenase (more precisely known as succinate ubiquinone oxidoreductase) (Figs. 1 and 2). Succinate (and α–glycerophosphate transferred from the cytosol to the matrix by the glycerol phosphate shuttle) alone reduces FAD to $FADH_2$, and each molecule furnishes two electrons to the respiratory chain, as do pyruvate and TCA-cycle intermediates isocitrate, α–ketoglutarate, and malate, by reducing NAD^+ to NADH.

Complex I is probably the major source of mitochondrial ROS under most physiologic conditions (335). Exogenous quinones can enhance ROS generation from isolated complex I (65). Inhibitors of complex I are useful for determining the source of ROS (96, 252). Several sites between the Flavin complex and the quinone site have been proposed to generate $O_2^{\bullet -}$ within complex I (77, 134, 160, 199, 216, 320). One or more of the Fe-S centers is a likely source (135, 160, 251), although the ubisemiquinone ($QH^{\bullet}$) binding site (143, 160, 200), or the flavin complex (216), *per se*, could also be sources, as depicted in Fig. 8. It has been proposed that complex I has two redox active nucleotide-binding sites; the F site is the location for electron entry for the NADH oxidation by Q, and coupled translocation of 4 H^+ for each NADH oxidized; the R site is where electrons exit during the succinate-supported NAD^+ reduction, which is $O_2^{\bullet -}$ generating (321).

When mitochondria oxidize succinate alone (lacking other TCA substrates), the energy of the $\Delta\mu H^+$ is used to transfer electrons

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against the redox potentials of the electron carriers from reduced coenzyme Q (quinol, QH_2) to NAD^+, rather than forward to the final electron acceptor O_2 (Fig. 9). This is called reverse electron transfer. Interestingly, a high transmatrix pH gradient appears to contribute more than the $\Delta\Psi_m$ to $O_2^{\bullet-}$ generation with succinate as the substrate (Fig. 10) (201). It was suggested that the ΔpH-sensitive $O_2^{\bullet-}$ generation is mechanistically linked to proton pumping at complex I (201). When electrons are transferred backward from complex II (22, 158, 307), this creates the largest source of $O_2^{\bullet-}$ as a percentage of O_2 consumption, although this does not likely occur *in vivo*. Reverse and forward electron flow is believed to contribute to an H_2O_2-production rate of ~400 and 50 pmol/min/mg protein, respectively (256).

Reverse electron transfer requires a large $\Delta\Psi_m$ or ΔpH; this occurs as electrons are passed to NAD^+ until the pool is fully reduced to NADH. $O_2^{\bullet-}$ generation by this mechanism ceases or decreases if the mitochondria are (a) uncoupled by a proton ionophore (*e.g.*, CCCP), (b) generating ATP during state 3 respiration, or (c) leaking protons into the matrix (matrix acidification or uncoupling proteins). Rotenone (Table 2), an irreversible inhibitor of electron transfer from the ubiquinone (Q) binding site to complex I, prevents reverse electron transfer and ceases $O_2^{\bullet-}$ release by this mechanism. This implies that $O_2^{\bullet-}$ is generated between the rotenone binding site and NAD^+ in complex I. The physiological importance of succinate only–induced $O_2^{\bullet-}$ generation by reverse electron transfer is dubious (335). However, because succinate is a TCA-cycle intermediate, it along with NADH-linked substrates likely contributes to $O_2^{\bullet-}$ generation during electron transfer *in vivo*, particularly if electron flow is impeded. It was reported that succinate concentration–dependent H_2O_2 generation was only slightly reduced in the presence of NADH-linked substrates (335) (*i.e.*, succinate-induced H_2O_2 production occurred under conditions of regular downward electron flow in complex I). Because NADH-linked oxidation was progressively decreased, but not abolished, by increasing succinate concentration, it was proposed that the two substrates compete for electron delivery to complex III, with succinate "pushing" electrons toward and NADH-linked substrates away from complex I (335). In this way, succinate concentration may modulate the rate of H_2O_2 release by controlling the $QH^{\bullet}/Q$ ratio.

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For forward electron transfer, it is postulated that the electrons from NADH-linked substrates (*e.g.*, pyruvate) are moved to ubiquinone Q *via* the flavin complex and Fe-S centers to form $QH^{\bullet}$, and in a second step that is linked to a low ΔpH, to form QH_2 (200). In the absence of rotenone, NAD^+- linked substrates can also enhance $O_2^{\bullet-}$ generation at complex I if the $\Delta\Psi_m$ is higher than normal (8); this condition of forward electron flow is favored by a high degree of reduction of the redox carriers proximal to the complex I proton pump. Once in a high redox state, $QH^{\bullet}$ can lose its unpaired electron to O_2 as all upstream redox centers are fully reduced (200). Moreover, if the ΔpH is large, $QH^{\bullet}$ is longer lasting, and more $O_2^{\bullet-}$ may be formed. It is not known whether complex I has a Q cycle (231) like complex III (200). $O_2^{\bullet-}$ is believed to be released only on the matrix side of the IMM at complex I (200, 256, 295).

F. Complex III (co-enzyme Q, bc_1 complex, ubiquinone/cytochrome c reductase)

This complex (Fig. 11) is believed to contain a Q cycle with an inner (Q_i) and outer (Q_o) pool of ubiquinone (Q) facing the matrix (**i**) and the intermembrane space (**o**) (42, 43, 97–99, 211, 268, 295, 296, 307, 308). Evidence for the Q cycle (231) arose when it was reported that addition of O_2 to anaerobic mitochondria caused a transient reduction of cytochrome *b* rather than an expected oxidation; this suggested that at least two different sites for electron transfer existed (43). In the subsequent model, $QH^{\bullet}$ is believed to form at both the Q_i and Q_o sites. First, ubiquinone (Q) is fully reduced to QH_2 in the inner side of the IMM and migrates to the outer side, releasing 2 H^+ and transferring one electron to cytochrome c_1 (Rieske Fe-S protein) to form the first $QH^{\bullet}$ and Q (the electron moves on to cytochrome *c* and cytochrome *c* oxidase (complex IV). The second electron reduces cytochrome *b* so that electrons are moved from Q at the Q_o site to the Q_i site, and Q is reduced to QH_2, completing the cycle. Cytochrome *c* and cytochrome *c* oxidase accept only single electrons in sequence. Thus, the complete reduction of Q at the Q_i site requires that the Q_o site must oxidize two QH_2 molecules in two successive turnovers. This powers the complex III proton pumps, but the second bifurcation reaction cannot occur unless the first does (138). Rather than transfer an electron to cytochrome c_1, the prolonged lifetime of $QH^{\bullet}$ is believed

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then to allow it to undergo autooxidation by releasing a singlet electron to be attacked by O_2, forming $O_2^{\bullet -}$.

Myxothiazol is a complex III inhibitor (296) that binds at the Qo site of the Q pool to block electron transfer from QH_2 at site Q_o to Fe-S clusters and to cytochrome b_2 (Table 2, Fig. 11). Stigmatellin blocks transfer of the first electron to the Fe-S center (center P, QH_2 oxidation site Q_o) (96). Antimycin A binds to the Q_i site to block electron transfer of the second electron to the Q_i site. Thus, antimycin A stimulates electron leak by inhibiting QH_2 formation (center N, Q reduction site) so that $Q^{\bullet}$ accumulates at the Q_o site, whereas myxothiazol prevents formation of $Q^{\bullet}$ at the Q_o site (Fig. 11). The finding that antimycin A did not affect H_2O_2 generation when added after myxothiazol indicated that myxothiazol stimulates H_2O_2 generation at a site in the Q_o center proximal to the site that is inhibited by antimycin A (296). Myxothiazol and stigmatellin inhibit the ROS-inducing effect of antimycin A, as noted earlier. The $QH^{\bullet}$ radical is fleeting and highly reactive and is not readily detected (320).

These studies suggested that $O_2^{\bullet -}$ generated at complex III would be released into the IMS rather than into the matrix space. Evidence for this was found in mitoplasts devoid of the OMM (152). This would further suggest that CuZnSOD is responsible for converting $O_2^{\bullet -}$ to H_2O_2 in the cytosolic space and that MnSOD would not have much importance for protecting the matrix from ROS damage. However, the Q_i site can also be a site for ROS generation, particularly with limited electron transfer into the Q_o center of complex III (263). Moreover, the MnSOD (matrix SOD) gene knockout is lethal (204, 214), whereas the CuZnSOD (extramatrix SOD) gene knockout is not, although the life span is shortened (118), and oxidative stress is elevated by twofold to threefold (235). This indicates the importance of high MnSOD activity to convert $O_2^{\bullet -}$ to H_2O_2, which unlike $O_2^{\bullet -}$, can easily exit the matrix. The relative amounts and conditions for ROS generated by complex III on either side of the IMM that play a physiologic role remains to be resolved. This information will be important because the balance between antioxidant capacities and $O_2^{\bullet -}$ generation within and outside the matrix that contribute to overall ROS emission is not clear.

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Most studies indicate that ROS are generated, especially at complex I, during state 4, when the redox state and $\Delta\Psi_m$ are high (Fig. 12), but not during state 3 (ADP phosphorylation). In the presence of succinate plus rotenone, $O_2^{\bullet-}$ can be generated by forward electron transfer *via* complex III, although at much lower rates than for reverse electron transfer with succinate alone, as noted earlier. This too requires a high redox state. However, it is possible to observe enhanced ROS at complex III with pyruvate/malate or succinate/rotenone during state 3 conditions (276) (high respiration, ATP synthesis, slightly decreased redox state, and $\Delta\Psi_m$) when O_2 availability is high (Fig. 13) or protein concentration is low (276), or when complex I is blocked with rotenone (199) (Fig. 14) and antimycin A is given to block the Q_i site (308). It is not known how substantial ROS are formed pathophysiologically (*i.e.*, hypoxia) if state 3 conditions are maintained, but small amounts of ROS clearly can initiate protective pathways, as discussed in sections VII and VIII.

IV. Pathologic Induction of Mitochondrial ROS Release

Tissue damage during hypoxia and reperfusion after ischemia has long been known to be associated with increased levels of various ROS (6, 15, 19, 123, 170, 290, 309, 318). Reperfusion after ischemia increases markers of ROS. Administration of scavengers of $O_2^{\bullet-}$ and H_2O_2, but not of $O_2^{\bullet-}$ alone, reduces the extent of injury (70). Isolated hearts exhibit a hypothermia-dependent linear increase in both $O_2^{\bullet-}$ (Figs. 6 and 15) and $ONOO^-$ (Fig. 7) (71). Although nonmitochondrial sources of ROS exist during ischemia [*e.g.*, NAD(P)H oxidase (141, 188, 328) and xanthine oxidase (25) in vasculature], the majority of ROS are likely derived from the mitochondrial respiratory complexes, as shown by the damage to respiratory complexes (212, 213, 245) and the effect of mitochondrial inhibitors (5, 28, 71, 81–86). The increase in H_2O_2 generation in mitochondria isolated after cardiac ischemia/reperfusion injury was enhanced by either rotenone or antimycin A (84); this suggested that both complexes I [Q and/or N_2 (final) Fe-S center] and III were damaged and capable of producing $O_2^{\bullet-}$ after ischemia/reperfusion injury. The source of $O_2^{\bullet-}$ generation during cell hypoxia leans toward complex III, in part because a decrease in oxidant stress during hypoxia in cytochrome *c*–null cells mimics the actions of myxothiazol and

stigmatellin (145, 146), which in effect prevent electron transfer to cytochrome *c* (Fig. 11). Loss of cytochrome *c* locks the Rieske Fe-S complex and cytochrome c_1 in a reduced state and prevents oxidation of QH_2 by the Fe-S complex to $QH^{\bullet}$ (145, 146).

A. ROS-induced ROS release

In addition to induction of ROS by hypoxia, ischemia, hypothermia, and mitochondrial toxins, ROS *per se* may lead to even greater ROS generation in a self-amplifying manner (46, 336). Photoexcitation of individual cardiac myocyte mitochondria caused an initial slow increase in ROS that culminated in a large burst of ROS (Fig. 16) that accompanied an abrupt loss of $\Delta\Psi_m$ and opening of the mitochondrial permeability transition (MPT) pore (336). This phenomenon, called ROS-induced ROS release (336), may be associated with Ca^{2+} overload and may play a role in initiating apoptosis, but whether normal stimuli initiate it is not known. It seems unlikely that Ca^{2+} overload can itself induce ROS generation because it dissipates $\Delta\Psi_m$ and reduces the redox state; but it may hinder the ROS scavenger system so that more ROS are liberated (8). During early ischemia in isolated hearts, ROS increase along with mitochondrial [Ca^{2+}] and the redox state (NADH), as shown in Fig. 17 (5, 70, 298), and treatment with exogenous intra and extramatrix ROS scavengers reduces ROS and mitochondrial [Ca^{2+}] and better maintains the redox state (NADH) on reperfusion after ischemia, as exemplified in Fig. 18 (70).

Another example is ROS generated by cytosolic NAD(P)H oxidase in a loop with ROS generation by mitochondria and then again by NAD(P)H oxidase (112). In this recent study, it was found that angiotensin II led to mitochondrial dysfunction by activating vascular NAD(P)H oxidase *via* a protein kinase C (PKC)-dependent pathway. The $O_2^{\bullet-}$ produced by NAD(P)H oxidase appeared to increase mitochondrial $O_2^{\bullet-}$ generation as well as to decrease $NO^{\bullet}$ bioavailability. Moreover, the H_2O_2 generated by mitochondria was proposed to cause a feed-forward activation of more $O_2^{\bullet-}$ by further activating NAD(P)H oxidase (112).

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B. ROS–induced Ca^{2+} loading

Because ischemia and hypoxia are marked in the cell not only by ROS release but also by Ca^{2+} loading, it is not clear whether one leads to the other or whether they are completely independent events (11). Again, it seems unlikely that increased Ca^{2+} can induce $O_2^{\bullet-}$ generation because it can also induce MPT pore opening to abolish $\Delta\Psi_m$ (59, 297). Ca^{2+}-transport systems are sensitive to redox conditions, so damage to Ca^{2+} import and export systems by ROS is more likely to lead to Ca^{2+} overloading (72). Moreover, ROS-triggered MPT pore opening is potentiated by Ca^{2+} overload (59). Nevertheless, it has been postulated that Ca^{2+} can induce ROS physiologically (59), a topic discussed in more detail in section VIII.

An effect of cytochrome *c* release during MPT pore opening and rupture of theOMMis the activation of caspase proteases. The collapse of $\Delta\Psi_m$ does not occur before these caspases are activated and released (267), and cytochrome *c* may function to shunt any free electrons released outside the matrix to complex IV, thus initially reducing $O_2^{\bullet-}$ generation (8). Activated caspase-3 can disrupt electron transfer at complexes I and III (but not at complex IV) to induce $O_2^{\bullet-}$ generation as a feed-forward pathway, leading to the collapse of $\Delta\Psi_m$ and initiation of apoptosis (267). This is discussed in more detail in section VII.

C. ROS generation during tissue ischemia and hypoxia

During the last decade, it has become increasingly clear that ROS are produced not only during reperfusion and reoxygenation after ischemia or hypoxia, but also during ischemia (Figs. 17–19) (5, 28, 70, 181–183, 247, 269, 270, 298, 317) and hypoxia (316). Figures 17 through 19 show examples of ROS fluctuations during ischemia and reperfusion during conditions of ischemia preconditioning, ROS scavenging, and hypothermia in isolated hearts. This seemingly paradoxic situation of ROS emission during ischemia (145, 146) can be explained because cells never truly become anoxic, and so O_2 remains available to form $O_2^{\bullet-}$ radicals (78). The rate of $O_2^{\bullet-}$ generation is chemically proportional by mass action to the O_2 concentration times the rate of electron leak (310), but in isolated liver mitochondria, an

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$[O_2]$ >50 μM does not alone result in greater $O_2^{\bullet -}$ generation, as shown in Fig. 20 (161). Mitochondria can respire normally at a very low p_{O2}; only when p_{O2} decreases to <5–7 torr does respiration begin to be limited by the O_2 supply (80). The K_m for O_2 of cytochrome *c* oxidase is <1 μM, and mitochondrial function is independent of a p_{O2} down to <2 torr. If O_2 levels are forced to decrease toward zero, electron transfer (respiration) through the respiratory complexes becomes markedly slowed, and the electron carriers operate at a more reduced state. How this occurs is not known, but it has been proposed (145, 146) that if the gene for cytochrome *c* expression were deleted, this would prevent cytochrome c_1 from giving up its electron at that site, and in turn, at the Reiske Fe-S protein site and in QH_2 at the Q_o site, so that the $QH^{\bullet}$ radical, and thus $O_2^{\bullet -}$ would not be generated, and these sites would remain locked in a reduced state.

During conditions of blocked electron transfer *via* the respiratory complexes and a highly reduced redox state (5, 70, 298), the $QH^{\bullet}$ radical may exist too long or be incapable of full reduction to QH_2 because of changes in protein conformation (61, 146, 221), so that electrons leak to combine with O_2 in a thermodynamically favorable reaction. Other possibilities are that a low $[O_2]$ at complex IV leads to cytochrome *c* reduction and so limits its capability to scavenge $O_2^{\bullet -}$ (63, 292), and that access of $QH^{\bullet}$ to O_2 is improved at low O_2 levels (146).

D. Very low p_{O2} and lack of mitochondrial ROS generation

Despite the accumulating evidence that ROS are indeed formed during ischemia and hypoxia, and mostly from the mitochondrial respiratory complexes, a recent direct study refutes that hypoxia enhances ROS. In recent, detailed experiments on this apparent paradox (161), it was demonstrated in liver mitochondria that between 20 and 200 μM O_2 concentration, respiration, H_2O_2 generation, and cytochromes a/a_3 redox state were unchanged; the H_2O_2 production rate was lower during state 3 than during state 4. Below 20 μM, $[O_2]$ respiration and H_2O_2 generation (Fig. 20), both decreased toward zero, and the cytochromes a/a_3 redox state became more reduced. It was of interest, however, that H_2O_2 generation as a *percentage* of the

respiratory rate increased fourfold (state 4) and sixfold (state 3) from 20 μM to near zero [O_2]. It was concluded (161) that (a) hypoxia in intact cells may elicit ROS from nonmitochondrial sources, (b) probes used are not specific for ROS, (c) cytosolic pathways are required for low O_2 sensing and ROS generation, (d) inhibition of complex IV by $NO^{\bullet}$ is required, (e) a hypoxia-induced decrease in tonal release of ROS downregulates signaling pathways involved with ROS, and (f) the fractional increase in ROS generation as a percentage of respiration acts as a hypoxia signal. It is, moreover, possible that hypoxia and ischemia produce derangements in mitochondrial electron transfer because of changes in substrate utilization (*e.g.*, succinate) and redox state that lead to generation of $O_2^{\bullet-}$ independent of the effect of a reduced [O_2] to accept electrons at complex IV.

Endogenous $NO^{\bullet}$ modulates respiration by its effect to compete reversibly for the O_2 binding site on cytochrome *c* oxidase (complex IV) (45, 58, 119). In endothelial cells, blocking the effect of $NO^{\bullet}$ to compete for O_2 at complex IV with an NOS inhibitor enhanced the rate of O_2 consumption (respiration) at very low O_2 concentrations (89). This indicated that $NO^{\bullet}$ inhibition of O_2 binding might be responsible for the inability of mitochondria to consume O_2 readily at low O_2 concentrations. Endothelial cells are a natural source of $NO^{\bullet}$, and $NO^{\bullet}$ may be generated in mitochondria (137, 139), and with $O_2^{\bullet-}$, to produce $ONOO^{-}$ (and ONOOH), which can modulate cell function, as addressed in section III. However, whether the enzyme $NO^{\bullet}$ synthase actually resides in the IMM is very controversial (54, 302).

Obviously, much work remains to de done to understand why, how, and from where ROS are generated in isolated cell or organ systems during hypoxia or ischemia but apparently not in isolated hypoxic mitochondria (161) without the use of electron-transport inhibitors. It is not known whether the ratio of $O_2^{\bullet-}$ generated from complex I and from the Q_i *versus* Q_o sites changes during hypoxia and reoxygenation or during ischemia and reperfusion, and how the activities of MnSOD *versus* CuZnSOD and other cellular antioxidant systems modulate the emission (generated minus scavenged) of distinct ROS. The specific sites, sidedness, relative amounts of $O_2^{\bullet-}$ generation, and its products in these complexes remain to be elucidated. It is possible that the source of $O_2^{\bullet-}$ generation and the

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particular kinds and amounts of ROS change during the course of ischemia and reperfusion, as suggested by the observed time-dependent phasic changes in NADH and FAD redox state, mitochondrial [Ca^{2+}], and $O_2^{\bullet-}$ levels (DHE, ETH) during ischemia (examples shown in Figs. 17 through 19) in isolated heart studies (5, 7, 70, 181, 183, 269, 298). Note, for example, in Fig. 19, that MnTBAP, given during brief ischemic pulses before the longer ischemic period, reversed the marked decrease in ROS during late ischemia and reperfusion induced by ischemia preconditioning (IPC), suggesting that $O_2^{\bullet-}$ is required to initiate IPC.

V. Antioxidant Defenses Against Pathologic ROS Formation

A. SODs, catalase, cytochrome c, GSH, and $TRXSH_2$, and other linked redox couples

Cellular antioxidant defenses depend on the reduction potential of the electron carriers and the reducing capacity of linked redox couples in the matrix ($NADH/NAD^+$ and $FADH_2/FAD$) and cytoplasm (10). Severe cell stress can induce ROS formation that exceeds the capacity of antioxidant enzymes, so that the net emission of ROS is increased. Up to a limit, mitochondrial and cell antioxidant systems are capable of neutralizing excess ROS. These include the intra- and extramatrix SODs (171, 263), and glutathione (GSH) (253, 279) and thioredoxin ($TRXSH_2$) (171) systems (Fig. 21), catalase in the cytosol, and cytochrome *c* in the intermembrane space (discussed in section VII). GSH, a tripeptide with the thiol (-SH) group of cysteine as the active site, is an abundant source of reducing equivalents; it reduces phospholipid hydroperoxides (PHPs) and H_2O_2, among other peroxides, *via* PHP glutathione peroxidase, an enzyme essential for life (8). GSH peroxidase, $TRXSH_2$, and $TRXSH_2$ reductase gene knockouts are also embryonically lethal (234). GSH and $TRXSH_2$ are maintained in a highly reduced state by their reductases; this allows them to reduce effectively H_2O_2 and lipid peroxides to H_2O. The resulting oxidized forms, GSSG (glutathione disulfide) and TRXSS (thioredoxin disulfide), rely on the $NAD(P)H/NAD(P)^+$ redox state to again become reduced.

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Thus, efficient mitochondrial bioenergetic function is required for the antioxidant activity of these systems.

B. Regulation of genes encoding mitochondrial antioxidant systems

The activities of antioxidant redox pairs do not appear to be constant. For example, a transcriptional coactivator, (PPAR)-γ coactivator 1-α (PGC-1α), a major regulator of oxidative metabolism and mitochondrial biogenesis, is believed to regulate the mitochondrial antioxidant defense system (153, 312). Endothelial cells that overexpressed PGC-1α upregulated the activity of oxidative stress–protective genes (mRNA for MnSOD, Prx3, Prx, $TRXSH_2$, $TRXSH_2$ reductase, UCP-2, and catalase) and resulted in reduced accumulation of ROS, increased $\Delta\Psi_m$, and reduced apoptotic cell death. This work (312) indicates that increased mitochondrial demand for energy production is met by activation of PGC-1α, which also enhances the mitochondrial antioxidant defenses.

C. ROS generation versus ROS scavenging

Normally, mitochondria likely serve as a net sink rather than a net source of ROS because of these very efficient scavenger systems (8), but ROS release can become excessive. During ischemia, $O_2^{\bullet -}$ and H_2O_2 levels increase as antioxidant defenses are overwhelmed and complex oxygen intermediates are formed (77, 240). One effect of an excessive initial release of ROS could be more ROS formation by ROS-induced ROS release by a mechanism that may involve MPT pore opening (336) or by caspase-induced modification of respiratory complexes during initiation of apoptosis (267). A high cytosolic GSH/GSSG ratio indicates a large reducing capacity to detoxify ROS and prevent activation of innermembrane anion channels (IMACs), which precedes MPT pore opening (10). The overabundance of ROS during Ca^{2+} overload with a collapsed $\Delta\Psi_m$ could reflect more the inability of the GSH, Pr-SSG, and $TRXSH_2$ systems to regenerate the reduced state (thus less H_2O_2 removal) than of more ROS to be generated (8).

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Superoxide, as a charged species, is relatively impermeable to membranes but can pass through anion (*e.g.*, IMAC) channels (11, 152). $NO^{\bullet}$ is not ionized and so is much more membrane permeable and can compete for $O_2^{\bullet -}$ with SOD to produce the nonradical $ONOO^-$ (29), which protonates at a low pH to ONOOH, which is highly reactive to tissues. $NO^{\bullet}$ can also react with O_2 to form nitrogen dioxide, $NO_2^{\bullet}$ (brown gas pollutant). The fate of $O_2^{\bullet -}$ (depending on $NO^{\bullet}$ availability) is typically dismutation to H_2O_2, which can then be converted to H_2O by catalase or to site-directed metal radicals [*e.g.*, $(Fe{=}O)^{2+}$] in the presence of transition metals bound within numerous organic molecules. It is the stable and membrane-permeable H_2O_2 that is the most abundant reactant that, in excess, likely leads to damage to cell structure and function *via* these ferryl reactants.

Because ''good'' ROS are regulators or modulators of normal cell function, it is difficult to draw the line on which ROS effects or amounts are beneficial and which are deleterious. The so-called diverse, non–receptor mediated, ''bad'' effects of ROS are mentioned here, but arbitrarily. It must be emphasized that the net effect of ROS is dependent not only on how much was produced, but also more important, on how much was not inactivated by antioxidant defenses. Thus, the generegulated GSH/GSSG, $TRXSH_2$/TRXSS, and Pr-SSG/PrSH redox system, as well as other scavengers that play important roles in protection against excess ROS-induced injury, are the same factors that probably modulate physiologic ROS signaling.

D. MPT pore opening and cytochrome c

Inhibition of MPT pore opening with cyclosporin A could prevent loss of the carrier cytochrome *c* so that an e^- is normally transferred to complex IV from cytochrome c_1, and $QH^{\bullet}$ is oxidized to Q rather than O_2 being reduced to $O_2^{\bullet -}$. As discussed also in section VII, MPT pore opening with release of oxidized [Fe(III)] cytochrome *c*, an $O_2^{\bullet -}$ scavenger, may reduce ROS emission in the IMS by oxidizing $O_2^{\bullet -}$. Inhibiting MPT pore opening may also prevent the loss of enzyme scavengers of ROS within the matrix that normally neutralizes ROS in the matrix (220). The independence or interrelation of matrix and extramatrix ROS release and scavenging and Ca^{2+} loading with MPT pore opening remains unclear.

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VI. Targets of Excess ROS Emission

A. DNA, proteins, and phospholipids

A sensitive target of excess ROS is mitochondrial DNA (Fig. 22), which is subject to an oxidation rate 10- to 20-fold higher than that of nuclear DNA (140, 142, 262). This is due in part to the lack of histone protection and the proximity to the ETS. For example, ROS intermediates can react with desoxyguanosine to form 8-hydroxydesoxyguanosine (288), which over time interferes with DNA duplication and RNA replication (125, 140). Mitochondrial proteins are also subject to attack by ROS (88, 121, 157, 257, 318). ROS can damage TCA-cycle enzymes, especially aconitase and α-ketoglutarate dehydrogenase (62, 168, 249, 250, 277). Other damaging effects of ROS are on the respiratory complexes, most at complex I and least at complex IV (66). Amino acids are also subject to oxidizing attack by ROS, and the GSH, $TRXSH_2$ and other redox systems are important not only for neutralizing ROS but also for repairing damage due to oxidation of proteins by virtue of their thiol groups (189). Protein oxidation can lead to unfolding and result in loss of catalytic function and degradation (289) [*e.g.*, ROS can damage the Na/K ATPase complex (287)].

Phospholipids are major targets of ROS (48); Transition metal radicals like $(Fe{=}O)^{2+}$ can initiate lipid peroxidation cascades in membranes to generate a complex mixture of short-chain aldehydes (Fig. 23), many of which are believed to be toxic. Accumulated ROS damage with aging may damage the lipid composition required for complex I activity. It was found (319) that liver mitochondria of old *versus* young rats showed a decrease in respiratory rate and reduced activity of complex I but not complex III. It was postulated that an increase in somatic mtDNA mutations would affect the hydrophobic subunits of complex I that are essential for CoQ binding and energy conservation, or that age-related defects of complex I, such as direct alterations of the protein or lipid environment, particularly cardiolipin, which is required for complex I activity, may play a role.

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B. Role of cardiolipin

Cardiolipin is a phospholipid found only in the IMM that anchors the mobile electron carrier cytochrome *c* to the IMM and optimizes the activity of electron-transport complexes, especially complex IV. Cardiolipin can be especially damaged by ROS because of these important roles (213, 258, 260, 261). Submitochondrial particles that exhibit loss of cardiolipin and cytochrome *c* during ischemia exhibit enhanced H_2O_2 release (83), which suggests their importance in protection against ROS. Excess ROS are primarily accountable for initiating MPT pore opening with the subsequent swelling, OMM rupture, and release of cytochrome *c* (187) and feedback generation of ROS (336). Accumulation of ROS over time is believed to be wholly or partially responsible for aging (210), but recently this has been questioned for vertebrates (234).

VII. Approaches to Reduce Excess ROS

A. Capacity of mitochondrial and cell reductants

From the foregoing material, it is evident that, to decrease ROS-mediated cell damage, MPT pore opening (35, 109, 110), and apoptosis due to hypoxia, ischemia, or toxins, one could attempt either to reduce the ROS generation or to enhance ROS scavenging so that overall ROS emission is diminished. Maintaining a large pool of reductants (like the GSH system) requires bioenergetically stable mitochondria to regenerate the reduced state after detoxifying the ROS. Supplying exogenous GSH may be protective but only if sufficient NAD(P)H is also available. Other cytosolic and matrix antioxidant systems exist, as described in section V. The endogenous mechanism of PGC-1α–mediated activation of redox cycling systems also was discussed in section V.

Chance *et al*. (77) proposed antioxidant capability must be found near sites of ROS production. An antioxidant mechanism, located at the site of electron transfer to complex IV and generation of $O_2^{\bullet -}$ in complex III, is carried out by cytochrome *c*. Cytochrome *c* can accept or donate an electron, depending on the valence of its heme (Fe) state. It was shown that adding exogenous cytochrome *c* to

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cytochrome *c*–depleted mitochondria reduced $O_2^{\bullet -}$ levels by sevenfold to eightfold (325, 332). In its reduced form (Fe^{2+}), cytochrome *c* normally generates a proton motive force ($\Delta\mu H^+$) during its oxidation by cytochrome *c* oxidase (complex IV), as one O_2 molecule is fully reduced to two molecules of H_2O by addition of $4e^-$ (8, 332). In its oxidized form [Fe^{3+}, or Fe(III)], cytochrome *c* forms O_2 and Fe^{2+} by virtue of the reducing ability (e^- donation) of $O_2^{\bullet -}$ with Fe(III), thus neutralizing superoxide. Interestingly, Fe^{2+} cytochrome *c*, but not Fe(III) cytochrome *c*, can suppress H_2O_2 levels by giving an e^- to H_2O_2 to form H_2O (332, 333). This is called the "alternative electron-leak pathway," (*i.e.*, reduction of preexisting H_2O_2 to H_2O by reduced cytochrome *c*). This mechanism can balance e^- leak to O_2 (forming $O_2^{\bullet -}$) with an e^- leak to H_2O_2 (forming H_2O) and so function to protect against too much ROS formation.

Because Fe(III) cytochrome *c* is a very efficient scavenger of $O_2^{\bullet -}$ within the matrix, when Fe(III) cytochrome *c* is released into the IMS during MPT pore opening and initiation of apoptotic signaling, it could act as an ideal extramatrix antioxidant (292). However, the loss of cytochrome *c* to the IMS may allow matrix $O_2^{\bullet -}$ to increase because of an absence of cytochrome *c* (220).

B. Exogenous SODs and catalase

Treatment with superoxide dismutases and catalase is another approach. However, effective delivery of these enzymes into the cytosol and matrix as a therapy is quite problematic. Overexpression of these enzyme systems could be successful in enhancing antioxidant defenses, but because ROS also play a significant physiologic role (described later), the effects may be deleterious, as indicated by a six- to 10-fold overexpression of MnSOD, which caused reduced fertility and abnormal development in mice (264), and the lack of any benefit to prolonging life span in mice overexpressing CuZnSOD (166). However, overexpression of catalase localized to mitochondria increased median life span in mice by 5 months (280). We reported that administration of the chemical SOD mimetic MnTBAP alone actually worsened cardiac function after ischemia in isolated hearts, whereas addition of glutathione and catalase with MnTBAP elicited the best protection (70); Fig. 18 shows the changes in ROS and

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mitochondrial Ca^{2+} during and after ischemia with these treatments. Our study suggested that enhanced generation of matrix $O_2^{\bullet-}$ during ischemia must be coupled with its dismutation to H_2O_2 and its oxidation to H_2O to offer the best protection.

C. Proton leak to modulate superoxide generation

Another perhaps more practical approach is to modify mitochondrial bioenergetics in a way that leads to reduced capability for producing $O_2^{\bullet-}$. As discussed earlier, it may be possible to generate $O_2^{\bullet-}$ during state 3 or when $\Delta\Psi_m$ is less polarized (291, 276, 324), but most experimental studies show that even a slight decrease in $\Delta\Psi_m$ (191, 201, 324) or transmatrix ΔpH (201) results in marked reduction or cessation of ROS production. Therefore, temporary partial uncoupling of respiration from phosphorylation by inducing an extrinsic proton ''leak'' may be therapeutic. Essentially, this process short-circuits the gated passage of H^+ through complex V to make ATP, so that respiration increases at the expense of no gain in phosphorylation (mild uncoupling) (47, 49, 60). It takes a greater electron-transfer rate to maintain the $\Delta\Psi_m$ when an H^+ leak is present. Oxidative phosphorylation is not wholly efficient, as up to 25% of basal H^+ flux into the matrix could be outside of complex V, as suggested by the heat produced as a fraction of the standard metabolic rate (Fig. 24) (55). The net effect of H^+ leak is to stimulate respiration (electron transfer) and produce heat at the expense of ADP phosphorylation.

H^+ leak is maximal when $\Delta\Psi_m$ is highly polarized, and little or no leak occurs when ATP is being generated. In vitro, state 4 respiration occurs only when no substrate ADP exists, ATP is not being consumed, or complex V is blocked (oligomycin). Thus, H^+ leak may be an intrinsic mechanism to attenuate electron leak and $O_2^{\bullet-}$ generation when the $\Delta\Psi_m$ is large. At a high $\Delta\Psi_m$ with a greater probability to generate $O_2^{\bullet-}$, the maximal capability to have a proton leak would tend to decrease $\Delta\Psi_m$ and thus $O_2^{\bullet-}$. The mechanism of basal H^+ leak is unknown but is thought to be protonophoric (*i.e.*, H^+ is carried across the IMM) (55). H^+ leak is assessed indirectly by any increase in respiratory rate or decrease in $\Delta\Psi_m$ or both during inhibition of complex V (Fig. 25).

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D. Uncoupling proteins

A small class of ''uncoupling'' proteins, called UCPs 1–4, are believed to induce an inward proton ''leak'' in charged mitochondria (49, 55, 176, 300). AMP may also act allosterically on adenine nucleotide translocase (aka adenine nucleotide transporter, ANT; or ADP, ATP carrier, AAC) to induce an H^+ leak (67). Proton leak could also be elicited by cycling of protonated/unprotonated nonesterified fatty acids (Fig. 24) (55, 132, 175), and by repetitive gating of the MPT pore by protons (53, 169). It is feasible that UCPs and AMP could play a role in protecting against ischemia and reperfusion injury, particularly if they could be activated or stimulated before the insult to reduce $\Delta\Psi_m$ and before the redox state increases because of inhibited electron transfer. Mitochondria isolated from perfused rat hearts subject to ischemic preconditioning had a greater H^+ leak than did ischemia controls (Fig. 25) (238); this H^+ leak was completely abolished by the UCP inhibitor GDP, or by the ANT inhibitor carboxyatractyloside (CAT) (238). ATP also inhibits UCPs (174). However, ANT, rather than UCP2, may be most responsible for the antioxidant mechanism in heart muscle mitochondria (238). It was suggested (238) that the smaller H^+ leak induced by ischemic preconditioning is mediated by UCP, because the H^+ leak in ischemia controls was blocked only weakly by GDP, but strongly by CAT, whereas the larger leak in ischemia/reperfusion alone is mediated by ANT.

E. HNE-induced proton leak

Some lipid peroxidation products, such as 4-hydroxy-*trans*-2-noneal (HNE) may induce partial uncoupling of mitochondria through UCPs and are thought to initiate protective mechanisms (41, 116). HNE can also induce uncoupling of oxidative phosphorylation by enhancing H^+ leak through other membrane proteins such as ANT if $\Delta\Psi_m$ is high (14). This H^+ leak, although preventable by CAT, did not interfere with ANT inhibition (14), so it was suggested that HNE causes a conformation change in ANT. Although ROS can activate UCPs (117) and ROS and HNE can directly cause a small proton leak (55), whether UCPs are activated by either endogenous ROS or HNE has been rigorously questioned (75). Because ubiquinone (Q) is not required for

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activation of UCPs (174), it is unlikely that ROS from complexes I or III exert a significant role. Together these studies indicate that mild uncoupling by H^+ leak reduces ROS formation in state 4 and that UCPs can induce a small H^+ leak, but evidence that mitochondrial ROS activate UCPs and the UCPs mediate protection against ROS-induced cell damage is not yet convincing.

It remains unclear how free fatty acids and alkenals like HNE activate proteins such as UCPs and ANT (14). One possibility is that a protonated fatty acid may cross the IMM into the matrix and dissociate, while the fatty acid anion is translocated back out of the matrix by the protein. Flip-flopping of the protonated and unprotonated forms would cause a net flux of H^+ into the matrix; other mechanisms are proposed (14). For alkenals (nonfatty acids), it was proposed recently that H^+ conductance *via* ANT occurs by formation of covalent adducts only at a high $\Delta\Psi_m$, thereby exposing sulfhydryl groups and lysine residues to attack by alkenals and causing a conformation change in ANT. This was based on the finding that CAT blocked H^+ leak *via* ANT if given before but not after HNE, whereas blockade of the ANT transport mechanism remained; this that implied HNE caused a permanent conformational change in ANT distinct from its translocation role (14).

F. ROS-induced proton leak

Brookes (55) proposed that ROS and H^+ leak comprise a loop not requiring UCPs to operate, but rather that the leak is dependent on the $\Delta\Psi_m$ alone. A high $\Delta\Psi_m$ would generate ROS (191), and the ROS would in turn induce an H^+ leak to reduce the $\Delta\Psi_m$ in a feedback manner. The generated $O_2^{\bullet -}$ could induce an H^+ leak indirectly through a lipid oxidation product or possibly *via* protonation of $O_2^{\bullet -}$ in the acidic intermembrane space to $HO_2^{\bullet}$ (215), which is membrane permeableand is deprotonated in the alkaline matrix (104). A decrease in the phosphorylation to respiration (P/O) ratio (*i.e.*, uncoupling), may come at the expense of higher $\Delta\Psi_m$ and ROS. The higher, but less efficient, metabolic rate associated with H^+ leak may be linked to the aging process (55). ROS could cause an H^+ "slip" rather than an H^+ leak. H^+ slip is described as direct reduction of cytochrome *c* by the $O_2^{\bullet -}$ generated into the intermembrane space, thereby bypassing the

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Q_i site (55), as discussed earlier in this section. Instead of $8H^+$ pumped per $4e^-$ at complex III, only $4H^+$ would be pumped per $4e^-$; this effectively represents a 50% reduction in ATP synthesis capacity at a given level of respiration.

VIII. Physiologic Modulation of Mitochondrial ROS Emission

A. H_2O_2 and $ONOO^-$ as chemical effectors

Despite rather efficient electron transfer along the respiratory system (*i.e.*, little electron leak), one might ponder why the mitochondrion and its surrounding environment are incapable of removing all the ROS that are produced despite the well-developed and necessary scavenging systems. Or one could argue that natural selection allowed a system that uses ROS for cell signaling and even protection against damage. Although the mitochondrion is largely a sink for $O_2^{\bullet -}$ and H_2O_2 due to its ROS scavenger systems, it likely releases small amounts of H_2O_2 as a natural chemical messenger in the modulation of mitochondrial and cellular function (27, 113, 126, 183, 203, 206). It was noted that the MnSOD knockout is lethal (204, 214), that MnSOD overexpression is associated with developmental abnormalities (264), and that ROS are required to trigger the apoptotic mechanism (68, 172, 207, 323), which is useful to actively eliminate poorly functioning cells and is required during embryonic development. It is now well known that ROS trigger or mediate ischemic and pharmacologic preconditioning of hearts (15, 27, 102, 181, 183, 248, 254, 303). However, it is difficult to determine how much, from where, and what kind of ROS is beneficial *versus* detrimental. Although a therapeutic goal may be to reduce ROS emission, particularly during oxidative stress, too much scavenging or the wrong kind of scavenging may eradicate protective cellsignaling mechanisms, as we have shown (70) and several reviews (118, 163, 203, 235, 303) have suggested.

The complex and extensive scavenging systems, the ROS/H^+ leak-feedback relation, and the possible role of UCPs in H^+ leak are several selected areas of research in which attempts have been made to understand the pathophysiologic regulation of ROS emission. It is evident that the bioenergetics state of mitochondria is important, not

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only for generation of ROS but also for ROS scavenging, and therefore the regulation of ROS emission. For example, free fatty acids function as natural mild uncouplers by preventing the transmembrane electrochemical H^+ potential difference (ΔmH^+) from being above a threshold critical for ROS formation by complexes I, III, or both (190).

$NO^\bullet$, a well-known modulator of cell function, is not reviewed in detail here because its generation within mitochondria is debatable, as mentioned in section IV. However, the interaction of $NO^\bullet$ and $O_2^{\bullet -}$ to form $ONOO^-$ and ONOOH as cell-toxic $NO^\bullet$ derivatives is well known; but low concentrations of $ONOO^-$ (0.1m*M*) were found to protect neurons against $NO^\bullet$-mediated apoptosis by activating the phosphoinositide-3-kinase (PI3K)/Akt antiapoptotic signaling pathway (106). The activation was accompanied by an increase in oxidized phosphoinositide phosphatase (PTEN), indicating that activation of PI3K/AKt inhibited PTEN and the $NO^\bullet$-mediated apoptotic pathway. $ONOO^-$ also was shown to stimulate pentose phosphate pathway (PPP) activity and the accumulation of NADPH, an essential cofactor for glutathione regeneration, and to activate glucose-6-phosphate dehydrogenase (G6PD), an enzyme that catalyzes the first rate-limiting step in the PPP (128). These and other studies [reviewed in (39)] indicate that low levels of $ONOO^-$, like low levels of H_2O_2, have a potential cytoprotective effect that could be explored therapeutically.

B. ROS modulation by cations

An additional area of focus recently is the role of cation exchangers and channels in modulating ROS and cell signaling. In developing the chemiosmotic theory of energy coupling, Mitchell (229) recognized that mechanisms must exist to exchange anions for OH^- and cations for H^+ in the IMM; otherwise, in creating the $\Delta\Psi_m$, the mitochondrion would swell and lyse, as only cations could leak in and anions leak out. Thus, mitochondrial cation antiporters (exchangers) were found to be necessary to regulate an osmotic differential across the IMM that would result from the high H^+ electrochemical gradient (34, 131). The chemiosmotic hypothesis requires several electroneutral cation antiporters for H^+ (NHE, Na^+/H^+ exchanger; and KHE, K^+/H^+ exchanger) and a low permeability to K^+ and Na^+ (34, 131, 239). In this way, countercations to H^+ would enter and leave the

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matrix along their electrochemical gradients, thus preventing osmotic swelling. Modulation of extramatrix and matrix pH affects the protonation of $O_2^{\bullet -}$ and $ONOO^-$, as discussed in section III.

C. K^+: A modulator of ROS generation?

The K^+ cycle (Fig. 26) is believed to be an important element in modulating mitochondrial function (34, 130, 131, 133) and may modulate ROS release (9, 159). The importance of regulated pathways for both K^+ uptake and K^+ efflux may be that this flux regulates a very fine tuning of mitochondrial volume that affects the rate of respiration (149, 150). K^+ may be taken up *via* one or more putative mitochondrial K^+ channels on a stimulus of low ATP levels or increased Ca^{2+} (K_{ATP}, K_{Ca}). In isolated cardiomyocytes, it was reported that increases in ROS and $NO^\bullet$ are downstream effects of application of a drug thought to open K_{ATP} channels (205), but we have found it difficult to observe significant increases in ROS with pharmacologic preconditioning agents, including K^+ channel openers and anesthetics in isolated hearts (182, 183, 298). Figure 27 (from ref. 182) shows a small increase in ROS (ETH) in isolated hearts exposed to sevoflurane that was blocked by MnTBAP but not by the putative K_{ATP} antagonist 5-HD.

D. Biphasic effect of K_{Ca} channels on ROS generation

We have postulated that mitochondrial K_{Ca} channels can modulate ROS production up or down by altering ΔpH independent of $\Delta\Psi_m$ (158, 159). We proposed that opening K_{Ca} channels with NS1619 produces a small H^+ leak (Fig. 28) (159). Others have proposed that K^+ cycling can account for no more than a small H^+ leak (49, 50). The increase in K^+ influx may be rapidly matched by K^+ efflux and H^+ influx *via* KHE driven by the pH gradient. As K^+ enters the matrix electrophoretically *via* K^+ channels (*e.g.*, during ischemia due to Ca^{2+} loading and low ATP during ischemia) K^+ may be exchanged for the energetically more favorable entry of H^+ (leak) because of the large ΔpH. If the inward H^+ leak is small, a small increase in proton pumping by complexes I, III, and IV enhances the respiratory rate without decreasing $\Delta\Psi_m$, while promoting ROS release (Fig. 29). K^+ flux could be a mechanism by which increased matrix Ca^{2+} modulates

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respiration.When the H^+ leak is large, $\Delta\Psi_m$ (and $O_2^{\bullet-}$ generation) would decrease, as discussed with other mechanisms of the H^+ leak (section VI).

If K^+ flux mediates an H^+ leak through KHE, then blocking KHE (quinine) should increase matrix K^+ but not H^+ levels. In preliminary experiments in isolated cardiac mitochondria, we observed that valinomycin, the putative K_{Ca} channel opener NS1619, and buffer Ca^{2+} increased matrix K^+ only in the presence of KHE blockade and that matrix pH decreased only in the absence of KHE (3, 4). A caveat to our work is that NS1619 has been reported (74) also to act as a protonophore, in which case, a small direct H^+ leak, rather than K^+ flux and KHE, might lead to the increase in H_2O_2 if $\Delta\Psi_m$ remains unchanged. Thus, although a large proton leak would be expected to act as a brake on further ROS generation, a small proton leak that stimulates respiration without reducing $\Delta\Psi_m$ may actually slightly increase ROS to modulate cell-signaling pathways. Moreover, in a recent study in which K^+ flux across the matrix was assessed indirectly by swelling and respiratory changes induced by valinomycin (K^+ ionophore) and nigericin (KHE activator), the putative K^+ channel openers NS1619 and diazoxide could not be shown to enhance K^+ flux (30).

E. K_{ATP} channel opening and ROS

A putative mitochondrial K_{ATP} channel opener was also reported to increase ROS production (Fig. 30), but by an alternative mechanism (9). In the presence of ATP, diazoxide increased ROS in isolated mitochondria, and the effect was inhibited by 5-hydroxydecanoate (5-HD); it was hypothesized that matrix K^+ influx causes matrix alkalinization, which retards electron transfer at complex I to cause electron leak and ROS formation (9).

The mitochondrial effects of drugs can be different, depending on the substrates used, their concentrations, and the bioenergetics state. For example, in isolated cardiac mitochondria, we found that two putative K_{ATP} channel openers (diazoxide and pinacidil) differentially attenuated mitochondrial respiration and that K_{ATP} channel antagonists (5-HD and glibenclamide) had no effect on this

(270). But when ATP synthase was inhibited by oligomycin, both K_{ATP} channel openers accelerated respiration, which was abolished by the K_{ATP} channel inhibitors. Many drugs also have biphasic effects, largely because they have concentration-dependent effects on mitochondrial energetics. For example, the various putative types of mitochondrial K^+ channel openers at low concentrations accelerate respiration and ROS generation without altering $\Delta\Psi_m$ or redox state (9, 159), whereas larger concentrations accelerate respiration even more, depolarize $\Delta\Psi_m$, and decrease the redox state and ROS generation (105, 130, 159). Moreover, these openers may have primary (74, 162) or secondary (159) effects to induce a proton leak into the matrix and may not actually open mitochondrial K^+ channels.

Thus, drugs that act on mitochondria can have biphasic as well as nonspecific effects, which complicate our understanding of mitochondrial function. As noted elsewhere, all these findings in isolated mitochondria are highly condition dependent and may not represent what occurs in intact tissue preparations. Further studies are necessary to determine the mechanism and extent to which specific mitochondrial K^+ channels do or do not modulate ROS at physiologic levels. That these channels are sensitive to either low ATP levels or high Ca^{2+} levels suggests that they could have important roles in mitochondrial regulation. Although many of the drugs used to assess ion channels in cell membranes may have specificity for a particular channel, the drugs used to assess mitochondrial channel function (*e.g.*, diazoxide or NS1619) do not appear to be specific for these channels but nevertheless exert protective function by an uncoupling mechanism and or by stimulation of ROS-dependent pathways.

F. Direct Ca^{2+}-induced ROS unlikely

Does Ca^{2+} directly modulate ROS? Ischemia and reperfusion result in increases in both Ca^{2+} and ROS, but it is unlikely that Ca^{2+} itself modulates ROS physiologically for the following reasons: (a) in pathologic situations, excess ROS results in Ca^{2+} loading, but not the other way around (72); (b) excess Ca^{2+} loading will itself cause MPT pore opening and dissipation of the $\Delta\Psi_m$ (72), but ROS-induced MPT pore opening does not require Ca^{2+} loading (186); (c) a Ca^{2+}-induced increase in oxidative phosphorylation (and decreased $\Delta\Psi_m$) tends to

decrease ROS, not to increase ROS (59, 72, 243, 296); (d) excess Ca^{2+} increases ROS only when mitochondrial inhibitors are used; otherwise, ROS decreases (59, 65); (e) blocking mitochondrial Ca^{2+} flux does not interfere with bursts of ROS produced by mitochondria during a local laser flash (11); and (f) $O_2^{\bullet-}$-induced mitochondrial swellingis Ca^{2+} independent, but Ca^{2+}-induced swelling is ROS dependent (127, 194). However, the possibility that Ca^{2+}-induced MPT pore opening, leading to loss of cytochrome *c*, differentially alters the matrix and IMS levels of ROS is discussed in section V.

G. Rate of oxidative phosphorylation and ROS generation

As discussed in section III, it is well known from O_2-consumption experiments in mitochondria that the state 3 to state 4 transition is usually accompanied by enhanced ROS production. Uncoupling (increased respiration without increased phosphorylation) typically leads to reduced ROS production, so it is possible that a change in the rate of oxidative phosphorylation modulates the rate of $O_2^{\bullet-}$ generation. Moderate increases in m[Ca^{2+}] induced by cell-receptor stimulation have long been thought to stimulate TCA-cycle substrate dehydrogenases and ATP synthase (108, 227, 228). However, it appears that this Ca^{2+}-induced stimulation is a slow process, because the dynamic, rapid stimulation of oxidative phosphorylation is not inhibited by partially blocking mitochondrial Ca^{2+} entry (148). Oxidative phosphorylation is activated with a time constant of seconds for the creatinine kinase system with an increase in ATP hydrolysis (313) that is too fast for Ca^{2+} to enter the matrix (52, 232). The prevailing view has been that oxidative phosphorylation is not governed by feedback control (17, 18, 51, 52, 180, 226). Computer simulations of increased cardiac work demands indicate that neither direct activation of ATP use alone nor a direct activation of both ATP use and substrate dehydrogenation, including Ca^{2+}-dependent TCA-cycle dehydrogenases, can account for the constancy of [ATP], [PCr] [Pi], and [NADH] during an increase in O_2 consumption in hearts in vivo (192, 193). Rather it was proposed that a so-called ''each- step-activation'' (or parallel-activation) mechanism is the explanation, in which all oxidative phosphorylation complexes are directly activated by some cytosolic factor related to muscle

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contraction in parallel with activation of ATP use and Ca^{2+}-dependent TCA-cycle dehydrogenases. This is a broad concept difficult to disprove. Others have proposed that oxidative phosphorylation in the heart is not likely regulated by quickly diffusing ADP and Pi in a simple feedback-control system (314). But another computer simulation (26), which incorporated each respiratory complex, substrate transporters including ANT, and cation fluxes, obtained reasonable fits to published data if P_i-dependent activation of dehydrogenase activity and the electron-transport system (especially complex III) were incorporated. Further development of this model (329) predicted that NAD is a more important regulator than ADP of the TCA-cycle dehydrogenases, and that a decrease in cytosolic pH decreases $\Delta\Psi_m$ and the ability to synthesize ATP.

Phosphorylation of complex IV at high matrix ATP/ADP ratios is known to decrease the H^+/e^- stoichiometry (H^+''slip'') of the complex inducing an intrinsic form of uncoupling in which the efficiency of proton pumping is reduced (see also section VII). Respiratory control is generally defined as the increase in respiration in the presence of ADP and its decline once ADP is phosphorylated. A possible mechanism for control of ROS release by oxidative phosphorylation may arise from a ''second mechanism of respiratory control,'' in which a high matrix ATP/ADP ratio is thought to allosterically inhibit complex IV (219). This inhibition of cytochrome *c* oxidase is switched on by cAMP-dependent phosphorylation and switched off by Ca^{2+}- activated dephosphorylation (179). cAMP-dependent phosphorylation of cytochrome *c* oxidase may optimize the efficiency of oxidative phosphorylation by maintaining a low $\Delta\Psi_m$ *via* the second mechanism of respiratory control. The effect of $NO^\bullet$ to compete with O_2 for binding at complex IV is discussed in section IV. Extrinsic uncoupling (H^+ ''leak'') of oxidative phosphorylation (*e.g.*, UCPs, HNE) is described in section VII.

IX. Role of ROS in Triggering or Effecting Cardioprotection

A. Pathways and mechanisms

Preconditioning is defined by the removal of the stimulus (brief ischemia or drug) some time before the onset of ischemia. Thus, the

stimulus does not directly induce the cardioprotection but rather some downstream factors do. Much circumstantial evidence suggests that mitochondria- derived ROS play an important role to initiate ischemic preconditioning (IPC) (Fig. 19) and pharmacologic preconditioning (PPC) (90, 100, 101, 217, 271, 306), which is effected by protein kinase pathways (223, 224, 247). Earlier studies examined the role of K_{ATP} channel openers and inhibitors to elicit and to block PPC. A common denominator of these studies was the finding that PPC, induced by the putative mitochondrial K_{ATP} channel opener diazoxide, could be blocked by ROS scavengers such as *N*-acetylcysteine (124) or *N*-mercapto-propionyl-glycine (254). A mild ROS stress, *per se*, triggered cardioprotection by activating protein kinases, but ROS also were reported to activate sarcolemmal K_{ATP} channels by modulating ATP binding at this channel, as this effect was blocked either by glibenclamide or by ROS scavengers (304, 305). Conversely, IPC requires ROS independent of K_{ATP} channel activity (120). Both K_{ATP} channel opening and H_2O_2 inhibit MPT pore opening (94). These and other studies suggested that a feedback loop may exist between K_{ATP} channels and ROS to produce cardioprotection (205, 237, 272, 298, 299), but much of this remains unresolved, in part because many of the drugs used as probes have other effects on cell or mitochondrial function (103, 154–156, 259). Kukreja (198) addressed the controversy in this area.

Mitochondrial Ca^{2+}-dependent K^+ channel (K_{Ca}) as well as K_{ATP} channel openers may play major roles not only in modulating mitochondrial bioenergetics but also in cardioprotection against ischemia/reperfusion injury. Most is reported about the putative K_{ATP} channel (1, 130, 131, 178, 195, 236). Mitochondrial K_{Ca} openers, like K_{ATP} channel openers, appear to mediate their effects through ROS-dependent mechanisms, as ROS scavengers, such as MnTBAP, block the protection (Figs. 18 and 19) (181, 183, 298). Figure 31 shows that paxilline (PX, an inhibitor of K_{Ca} channels) and MnTBAP (TB, an $O_2^{\bullet -}$ dismutator) blocked the ROS (DHE) and redox (NADH) reducing effect of NS119 (putative K_{Ca} channel opener) during ischemia and reperfusion of isolated hearts. Diverse cardiac-preconditioning drugs given before ischemia and reperfusion in isolated hearts result in improved function along with improved tissue redox state (NADH and FAD), reduced cytosolic and mitochondrial Ca^{2+} loading, and reduced

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production of $O_2^{\bullet -}$ and $ONOO^-$ during ischemia and reperfusion (76, 181, 248, 298). If the observed changes in isolated mitochondria by mild K^+ flux that promotes the increase in $O_2^{\bullet -}$ (observed *via* dismutation to H_2O_2) also occur in vivo, then modulation of mitochondrial bioenergetics by K^+ flux might ultimately be a key initiator of mitochondrial and cell protection.

B. Inhibiting complex I and cardioprotection

Another approach to protect cells undergoing ischemia is to inhibit complex I during ischemia (81). Rotenone or amobarbital (a reversible inhibitor of complex I) given just before the onset of cardiac ischemia resulted in improved oxidative phosphorylation and retention of cytochrome *c* in mitochondria isolated during ischemia or on reperfusion (82, 85). We found subsequently that amobarbital, *per se*, when given for 1 min before ischemia, arrested hearts, increased $O_2^{\bullet -}$ emission (and NADH, with no change FAD), and reduced mitochondrial $[Ca^{2+}]$ (Fig. 32) (5); this led to reduced $O_2^{\bullet -}$ generation and mitochondrial $[Ca^{2+}]$ during ischemia and reperfusion. These *ex vivo* results suggested that amobarbital blocks electron transfer upstream of the rotenone binding site but distal to the NAD+ binding site. During reperfusion after ischemia, we observed a more reduced NADH redox state, decreased $O_2^{\bullet -}$ generation, and reduced mitochondrial $[Ca^{2+}]$ in this intact heart model (5). These mitochondria-protective effects were accompanied by improved cardiac function and smaller infarct size.

X. Regulation of Cellular Processes by Mitochondria-Derived ROS

Intracellular signaling pathways (*e.g.*, Ca^{2+}, cAMP, protein phosphorylation–dephosphorylation cascades) are essential for modulating cell metabolism and function. Many of these pathways are linked to extracellular receptor ligands at the plasma membrane, but many are derived from within the cell (*e.g.*, the feedback link between the cell's metabolic rate and mitochondrial respiratory activity). Accumulating evidence indicates that ROS are not always deleterious but are essential participants in cell signaling (113, 126, 163, 303). Whether ROS are ''bad'' or ''good'' could simply be a matter of quantity or rate of production, or it may also depend on the specific species,

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the site of generation, and the site of ROS release. The important and well-examined role of non–mitochondriaderived ROS ($O_2^{\bullet -}$ and $NO^{\bullet}$) in vascular sensing and control (328) is not addressed here. The "pathologic" roles of ROS and Ca^{2+} loading during cellular stress [*e.g.*, ischemia, hypoxia that lead to induction of cell necrosis and apoptosis (68, 207, 323, 330)] were addressed in section III.

A. Cell signaling by oxidative modifications and redox systems

ROS-induced cell signaling involves two general mechanisms: alterations in the intracellular redox state (*e.g.*, GSH, $TRXSH_2$, and other redox systems) (Fig. 21) and oxidative modification of proteins (92, 303). ROS appear to regulate a large number of signaling pathways that modulate cell function, but the actual molecules targeted by the ROS are unclear in most cases. Evidence for a physiological role of ROS emission are based in part on the effects of antioxidants and inhibitors of ROS generation to prevent growth factor and cytokine-activated signals, or some other physiological effect, and the effects of exogenously applied oxidants to activate these responses.

Synchronized oscillations in $\Delta\Psi_m$, the redox states of NADH, GSH/GSSG and other redox pairs, and ROS can occur across isolated cardiomyocytes (11) and were postulated to result from the balance between $O_2^{\bullet -}$ efflux through IMM anion channels (IMAC) and the intracellular ROS scavenging capacity. This was based on a computational model (93) of mitochondrial energetics and Ca^{2+} handling that reproduced the observed oscillations, which could be modulated by ROS scavengers or by the rate of oxidative phosphorylation. Oxidation of thiol groups appears to govern the sequential opening of IMAC (before MPT pore opening occurs), based on the GSH/GSSG redox status (10). Such oscillations may play a role in physiologic timekeeping or redox signaling across the cell or both.

B. Examples of signaling by ROS

The role of ROS in initiating preconditioning protection, particularly well known in the heart, is a vivid example of how ROS

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modulate cell signaling by mitochondrially generated ROS. To be a modulator of physiological function, the oxidizing effect of ROS should be transient and reversible, generally by other reducing agents. Broad examples of ROS (typically H_2O_2)-induced signaling (266) are inhibition of tyrosine phosphatase (278, 315) leading to cell proliferation; translocation and activation of serine/threonine kinases, such as protein kinase C (247, 301) and tyrosine hydroxylase mRNA, the rate-limiting enzyme in catecholamine biosynthesis (196); activation of the MAPK family of protein kinases (including MAPKs ERK, JNK, p38) that mediate mitogen and stress-activated signals; NF-κB, a transcription factor that regulates gene expression involving immune and inflammation responses; and AP-1, a transcription complex (303).

One example of a regulatory feedback loop between ROS signaling and enzyme modification is the following: pyruvate was shown to activate JNK1 indirectly *via* pyruvate-induced mitochondrial H_2O_2 release (242); in turn, JNK1 inhibited the activity of the metabolic enzymes glycogen synthase kinase 3β, allowing increased activity of glycogen synthase so that glucose was stored as glycogen rather than undergoing glycolysis. Thus substrate metabolism can be tied directly to mitochondrial ROS. Ion-channel function is also subject to modification by thiol reducing and oxidizing agents (73, 163, 164).

C. Importance of cysteine thiols in ROS-induced signaling

How ROS modify the structure and function of these and other signaling proteins is not well understood, but several mechanisms are described (163, 303), particularly those involving the key thiol groups in regulatory proteins (20). One is the reversible oxidation of the sulfhydryl group (—SH) in the cysteine residue to form SOH, SO_2H, or SO3H derivatives that alter the activity of an enzyme if the cysteine is located in a catalytic domain or DNA-binding site. For example, H_2O_2-induced oxidation of a cysteine residue located in its catalytic site precisely and rapidly inhibits tyrosine phosphatase (278, 315). The modification of –SH to $–SO_2H$ prevents the enzyme from further oxidation and promotes reversibility to its active form (except for SO_3H) by thiols (278, 315).

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Another mechanism for posttranslational protein modification by ROS (Fig. 7) is a conformational change in the structure of protein kinases by formation of intramolecular disulfide bridges in cysteine linkages. Another is protein dimerization by intermolecular disulfide linkages of monomers to form an active protein, or dissociation of an inactive protein complex linked by disulfide linkages into an active protein. Several proteins can also become cross-linked because of active dityrosine formation from two tyrosine molecules by H_2O_2 peroxidase–dependent reactions. Mixed-function oxidases can facilitate metal-catalyzed oxidation by $O_2^{\bullet -}$ of proteins with 4Fe-4S tetranuclear cores to mark them for ubiquitination and so alter their stability (303). To qualify as cell-signaling pathways, these oxidation reactions should be reversible by using the cell's redox systems (*e.g.*, GSH and $TRXSH_2$), as discussed previously.

D. ROS oxidation reactions

Precursor ROS do not always oxidize proteins directly but rather through an oxidized and reactive phospholipid intermediary (Fig. 23). Oxidized lipids can activate cellsignaling pathways by non-covalent bonding to a receptor, by covalent binding with direct modification of the protein, and by activating pathways that induce ROS formation from other sources (334). The electrophilic lipid peroxidation product 4-HNE (section VI) causes ROS emission that activates MAPK pathways (311), and 4-HNE selectively inactivates thiol containing proteins, such as a-ketoglutarate dehydrogenase and pyruvate dehydrogenase, and thus inhibits NADH-dependent respiration (complex I) (168).

E. O_2 sensors

A physiologic response to low O_2 requires an O_2 sensor coupled to a signal-transduction system (80). For example, ATP synthesis is tightly proportional to O_2 consumption in the presence of adequate O_2, so the ability of the cell to sense a critically low level of O_2 would appear to be fundamental to restoring O_2 levels for ATP synthesis (45). But the molecular identity or mechanism of an O_2 sensor has not been forthcoming. Several O_2-sensor mechanisms have been proposed and reviewed (80): O_2-sensitive heme proteins; O_2-sensitive ion channels;

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O_2-sensitive NADPH oxidase; O_2-sensitive complex IV; and O_2-sensitive ROS generation. Many of the proposed O_2 sensors do not appear to have the sensitivity to act directly as O_2 sensors, but they could all be linked to hypoxia-induced changes in redox state or ROS generation. For example, the capacity of complex IV to produce H_2O from O_2, electrons, and protons is not impaired at even very low p_{O2} values, but enzyme activity (V_{max}) is reduced so that reduced electron transfer and a more reduced redox state ensue (80).

F. Hypoxia-inducible factors

A principal regulator of the response to hypoxia is the hypoxia-inducible factor (HIF) family of transcription factors (31, 61, 115, 165, 177, 185, 221, 282–286, 316, 327). Although constitutively expressed, the HIF-1α subunit has a very short half-life in normoxic conditions because of its rapid hydroxylation by prolyl-4 hydroxylases (PH1–3) (12, 36). During hypoxia, the HIF-1α subunit becomes uninhibited by PHs, so that HIF-1α accumulates and transfers to the nucleus to initiate HIF-1α-mediated transcription of more than 70 genes involved in protein stability, such as heat-shock proteins (HSPs) (40). Therefore, low O_2 would appear to control activation of HIF-1α by inhibiting PH activity. The mitochondrion would seem to be a natural sensor for O_2 because complex IV is where O_2 binding and O_2 consumption occur. However, blocking complex III with antimycin A and cyanide to block complex IV did not inhibit the HIF-1α in isolated cardiomyocytes in response to hypoxia (79). Moreover, inhibition of PH did not occur until O_2 levels were below 5%, with maximal activation at 0.5% O_2 (177). Rather than O_2 being the actual sensor, it was proposed that the actual sensor is the redox changes upstream from complex IV (146).

The signaling consequences of hypoxia are probably modified by $NO^{\bullet}$. Nitric oxide, and other inhibitors of mitochondrial respiration, can prevent stabilization of HIF-1α during hypoxia as a result of an increase in PH-dependent degradation of HIF-1α (147). During inhibition of mitochondrial respiration by hypoxia, O_2 was found to redistribute toward nonrespiratory O_2-dependent targets such as PHs, so that they no longer registered hypoxia (147). It was concluded that $NO^{\bullet}$ acts as an endogenous regulator of intracellular O_2 availability;

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inhibition of mitochondrial O_2 consumption may create a paradox of increased O_2 availability for prolyl hydroxylation of HIF-1α, so that the cell may fail to register hypoxia. In a related study (222), lower concentrations of $NO^{\bullet}$ (<400 n*M*) caused a rapid decrease in HIF-1α stabilized by exposing cells to 3% O_2. This effect of $NO^{\bullet}$ was dependent on inhibition of mitochondrial respiration, because the $NO^{\bullet}$ effect was mimicked by other inhibitors of mitochondrial respiration, including those not acting at complex IV. It was suggested that although stabilization of HIF-1α by high level of $NO^{\bullet}$ may have pathologic consequence, the inhibitory effect of low levels of $NO^{\bullet}$ may be physiologically relevant.

G. ROS as O_2 sensors

It has been suggested that O_2 sensing by the cell is actually carried out by mitochondrial ROS (31, 61, 80, 146, 221), including $NO^{\bullet}$ (57). One proposal is that the increase in mitochondrial redox state secondary to the reduced V_{max} of complex IV during hypoxia creates a more favorable condition for $O_2^{\bullet -}$ generation and downstream ROS production (Fig. 33) (80). Thus, the common denominator for O_2 sensing by ROS would be the heightened redox state and $\Delta\Psi_m$ or both. However, the mechanism for the reduction in V_{max} by low O_2 levels is not known.

Support for a role of ROS in HIF-1α stabilization derives from studies showing that the absence of cytochrome *c* or blocked Rieske Fe-S proteins under hypoxic conditions blocks HIF-1α stabilization (61, 221). How H_2O_2 inhibits PH activity is not yet known, but likely involves one of the ROS- induced protein modifications discussed earlier. When iron chelators were given to block Fe^{2+} from reacting on PH, or HIF-1α hydroxylation was prevented by 1-dimethyloxallyl glycine (DMOG) to compete at PH, HIF-1α accumulated and induced gene expression (173). These mechanisms depend on the observed increase in mitochondrial ROS in cells when O_2 levels decrease during hypoxia and ischemia. However, this must be reconciled by the finding in isolated mitochondria that hypoxia does not itself increase ROS (Fig. 20) (161). Thus, it has been proposed that the hypoxic signal is a decrease in ROS, not an increase (13, 184, 233, 326). Studies in intact cells, tissue and isolated hearts indicate that ROS emission increases

during hypoxia and ischemia, as discussed earlier (section III), so the controversy on the actual hypoxia-sensing mechanism continues.

H. Peroxide-induced TCA shunts

Peroxides produced by mitochondria can create nonenzymatic shunts in the TCA cycle (56, 121). Succinate dehydrogenase (complex II) is very resistant to oxidation by H_2O_2 or *tert*-butyl hydroperoxide (*tert*-BuOOH) (250), but α-ketoglutarate dehydrogenase activity is markedly decreased (168, 250) because of its susceptibility for modification by peroxides of its thiol groups (167, 249). Although this decreases the enzymatic conversion of α-ketoglutarate to succinyl Co-A and succinate by decarboxylation and blocks production of reducing equivalents (NAD+ to NADH) at this step, these oxidants nonenzymatically (chemically) decarboxylate α-ketoglutarate to succinate and thus reduce the level of peroxides (Fig. 34) (121). This bypass depends on a supply of α-ketoglutarate by transamination because its precursor, aconitate, is also inactivated by peroxides (62, 249). This TCA bypass increases the contribution of succinate to the total energy supply ($FADH_2$ dependent) while decreasing the NADH-dependent energy supply (168). Because succinate can contribute to reverse electron transfer and generation at complex I under certain conditions, control of succinate levels during acute cell stress may be beneficial. Another peroxide bypass, the electron-leak pathway mediated by cytochrome *c* (332, 333), was discussed in section VII.

Hypoxia, increased succinate, and decreased aketoglutarate can each lead to inhibition of PH (208, 281), which permits translocation of HIF-α to the nucleus to support HIF-α–dependent transcription of the multitude of genes responsible for O_2 transport, vascularization, and anaerobic energy production (208). Addition of succinate improved oxidative phosphorylation after ischemia/reperfusion injury (69), so the role played by succinate is not well understood. These studies again point to H_2O_2 (and other peroxides), rather than $O_2^{\bullet -}$ *per se*, as the transduction factor in physiologic regulation by ROS (61). Modulation of a key TCA intermediate, in this case succinate, by ROS is a prime example for demonstrating how mitochondria and cells respond to oxidative stress by altering gene expression. If ischemia or hypoxia induces reverse electron transfer by a TCA shunt to the FAD-

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linked substrate succinate because of inhibition of the NADH-linked substrates, the resulting increase in $O_2^{\bullet -}$ generation at complex I may signal measures to alter mitochondrial function. It was noted earlier that inhibiting complex I with amobarbital improved cardiac function after ischemia and reperfusion and improved mitochondrial function (5, 85). Thus, it is possible that reverse electron transfer may be, at least in part, responsible for some of the increase in ROS during ischemia. As discussed in section III, NADH-linked substrates do not appreciably block succinate-induced H_2O_2 emission via reverse electron transfer (335).

XI. Summary

A. Difficulties in understanding the role of ROS

Although H_2O_2, and in some situations, $O_2^{\bullet -}$ (21), are found to trigger a multitude of signaling pathways (most unknown) *via* their reactants such as peroxides, the precise mechanisms by which mitochondrial ROS initiate the signal-transduction pathways are mostly unknown, despite the TCA succinate bypass example given earlier. One difficulty in a better understanding of ROS modulation of cell signaling is the short half-life and reactive state of many ROS. The source or compartmentalization of the ROS is difficult to discern in most situations. The original oxidation product may itself be oxidized to a product that is the active signaling molecule. A surge in ROS or changes in redox capacity may have local or broad effects on many regulatory proteins simultaneously, so determining specificity is difficult. The ROS can originate from extracellular, cytosolic, or a nonmitochondrial organelle, or from a combination of extra- and intramitochondrial compartments. Moreover, redox regulation of ROS occurs at multiple levels. All of these complicating factors make it quite difficult to sort out the exact signaling mechanisms of ROS. It would appear that Nature has developed a highly sophisticated system to regulate specifically the generation and scavenging of ROS and to modulate the downstream effects of physiologically induced ROS emission on mitochondrial and cell activity.

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B. Future directions

From this review, it is clear that much has been learned about mitochondrial sources of ROS, the mechanisms of ROS generation and scavenging, and the pathologic effects of ''bad'' ROS on cell processes. However, knowledge of the source, amount, generation site, and specific ''good'' reactive species that are involved in cellular and organelle protection and physiologic modulation of cellular activity, even in one cell type (*e.g.*, the cardiac myocyte), remains in its infancy. We hope that future studies will refine and detail our specific understanding of cell-signaling pathways mediated by mitochondrial ROS by using more physiologic approaches in normal cell settings. From these studies, it is hoped that novel therapeutic targets and drugs can be discovered by which to treat mitochondrial and cellular stresses, or at least to assist the organism's intrinsic protective mechanisms.

Footnotes

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Abbreviations

2-OH-E+, 2-hydroxyethidium; 5-HD, 5-hydroxydecanoic acid; $\Delta\mu H^+$, proton motive force, transmembrane electrochemical H^+ potential difference; $\Delta\Psi_m$, mitochondrial transmembrane potential; ΔpH_m, mitochondrial pH gradient potential; ANT, adenine nucleotide translocase; CAT, carboxyatractyloside; CCCP, carbonyl-cyanide-*m*-chlorophenylhydrazenone; DCF, 2'7'-dichlorofluorescein; DHE, dihydroethidium; DMOG, 1-dimethyloxallyl glycine; ETS, electron-transport system; $FADH_2$, flavin adenine dinucleotide (reduced); GSH, glutathione (reduced); H_2O_2, hydrogen peroxide; HIF,

hypoxia inducible factor; HNE, 4-hydroxy-*trans*-2-noneal; IMAC, inner membrane anion channel; IMM, inner mitochondrial membrane; IMS, intermembrane space; IPC, ischemic preconditioning; K_{ATP}, ATP-sensitive K^+ channel; K_{Ca}, Ca^{2+}-sensitive K^+ channel; KHE, K^+/H^+ exchange; L-NAME, N^G-nitro-l-arginine methyl ester; MAPK, ERK, JNK, p38, family of protein kinases; MnTBAP, Mn(II)tetrakis(4-benzoate) porphyrin chlorine; MPG, *N*-(2-mercaptopropyonyl)glycine; MPT, mitochondrial permeability transition; NADH, nicotinamide adenine dinucleotide (reduced); NHE, Na^+/H^+ exchange; $NO^\bullet$, nitric oxide radical; $O_2^{\bullet-}$, superoxide anion radical; OMM, outer mitochondrial membrane; $ONOO^-$, peroxynitrite; PH, prolyl-4-hydroxylase; PHP, phospholipid hydroperoxide; PPAR-γ, peroxisome proliferator activated receptor gamma; PPC, pharmacologic preconditioning; Prx3, Prx5, peroxirredoxins 3 and 5; Pr-SSG, glutathione-protein mixed disulfides (oxidized); Q, coenzyme Q_{10}, ubiquinone, quinine; ROS, reactive oxygen species; SOD, superoxide dismutase; TCA, tricarboxylic acid; $TRXSH_2$, thioredoxin (reduced); UCP, uncoupling proteins.

Disclosure Statement

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Fig.1.

Schema of electron transfer through respiratory chain with sites of ROS ($O_2^{\bullet -}$) generation at complexes I and III. Electron transfer is reversible, except at complex IV, and forward transfer results in extramatrix proton pumping at complexes I, III, and IV, with reentering of protons at complex V coupled to ATP synthesis. Succinate can lead to reverse electron transfer, reduction of NAD^+ to NADH, and $O_2^{\bullet -}$ generation at complex I. [Used with permission and modified from Batandier *et al*. (22)].

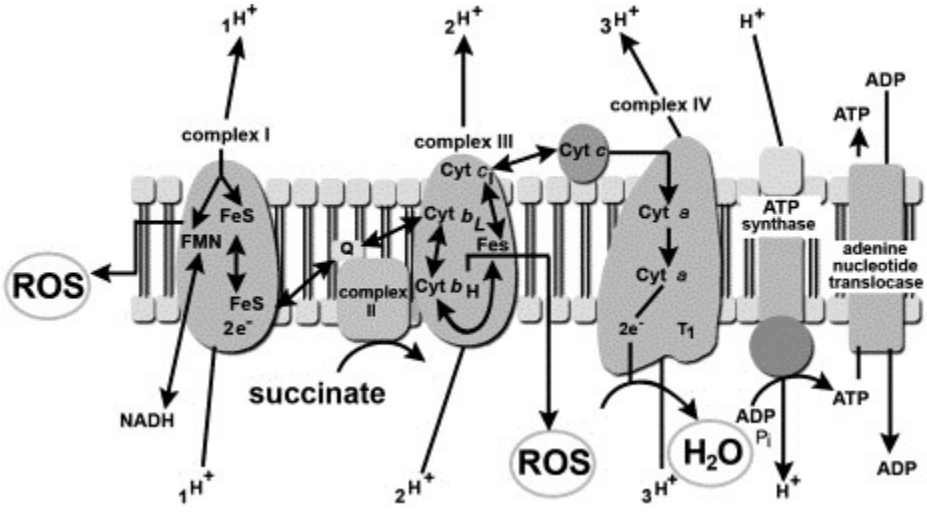

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Fig. 2

(A) Sites of $O_2^{\bullet -}$ generation along electron-transport system with several respiratory inhibitors. (**B**) Diagram of electron-transport system with standard reduction potentials ($E^{o'}$) of mobile components and $\Delta E^{o'}$ where sufficient free energy is harvested to synthesize ATP.

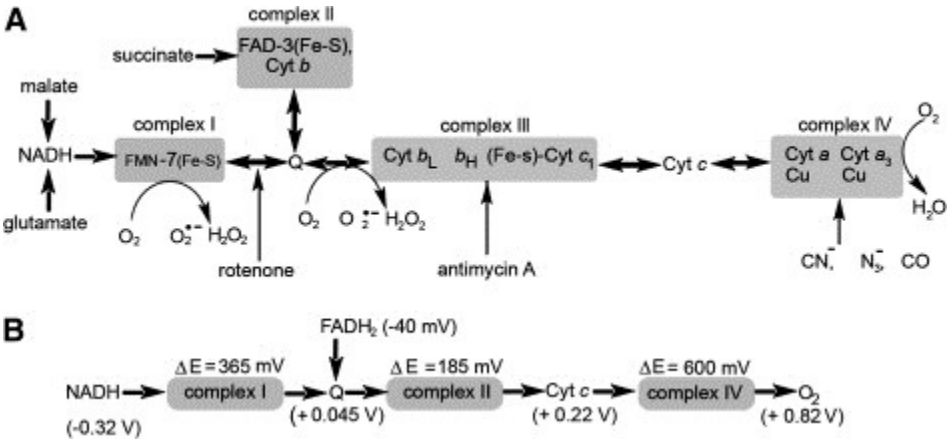

Table 1.

Examples of Reactive Oxygen (Nitrogen, Chlorinating) Species

Radicals	***Nonradicals***
Superoxide, $O_2^{\bullet -}$	Hydrogen peroxide, H_2O_2
Ferryl, Fe(IV) = $O^{\bullet}$	Hypochlorous acid, HOCl
Peroxyl, $RO^{\bullet}$	Oxone, O_3
Alkoxyl, $RO^{\bullet}$	Singlet oxygen, $^1\Delta_g O_2$
Nitric oxide, $NO^{\bullet}$	Peroxynitrite, $ONOO^-$
Hydroperoxyl, $HO_2^{\bullet}$	Peroxynitrous acid, ONOOH

Note that not all radicals ($O_2^{\bullet -}$) or nonradicals (H_2O_2) are highly reactive, whereas other radicals and nonradicals are highly reactive ($HO_2^{\bullet}$, ONOOH). Carbon-centered radicals ($Cl_3C^{\bullet}$), sulfur-centered radicals ($RS^{\bullet}$/$RSS^{\bullet}$), and nitrogen-centered radicals ($C_6H_5N = N^{\bullet}$) also exist (151).

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Fig. 3

The products of superoxide ($O_2^{\bullet -}$) and their catalysts.

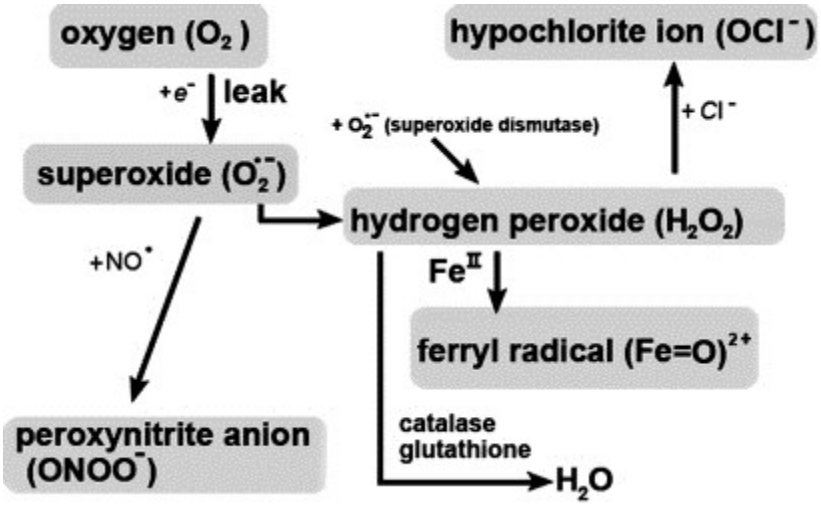

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Fig. 4

EPR spectra indicating formation of $O_2^{\bullet -}$ (DMPO-OH signals) by mitoplasts. The $O_2^{\bullet -}$ spin-trap DMPO and antimycin A (**B**) are necessary to observe the signals (Control; **A**), which are abolished by SOD (**C**). Succinate + antimycin A (**D**). [Reprinted with permission from Han *et al*. (152)].

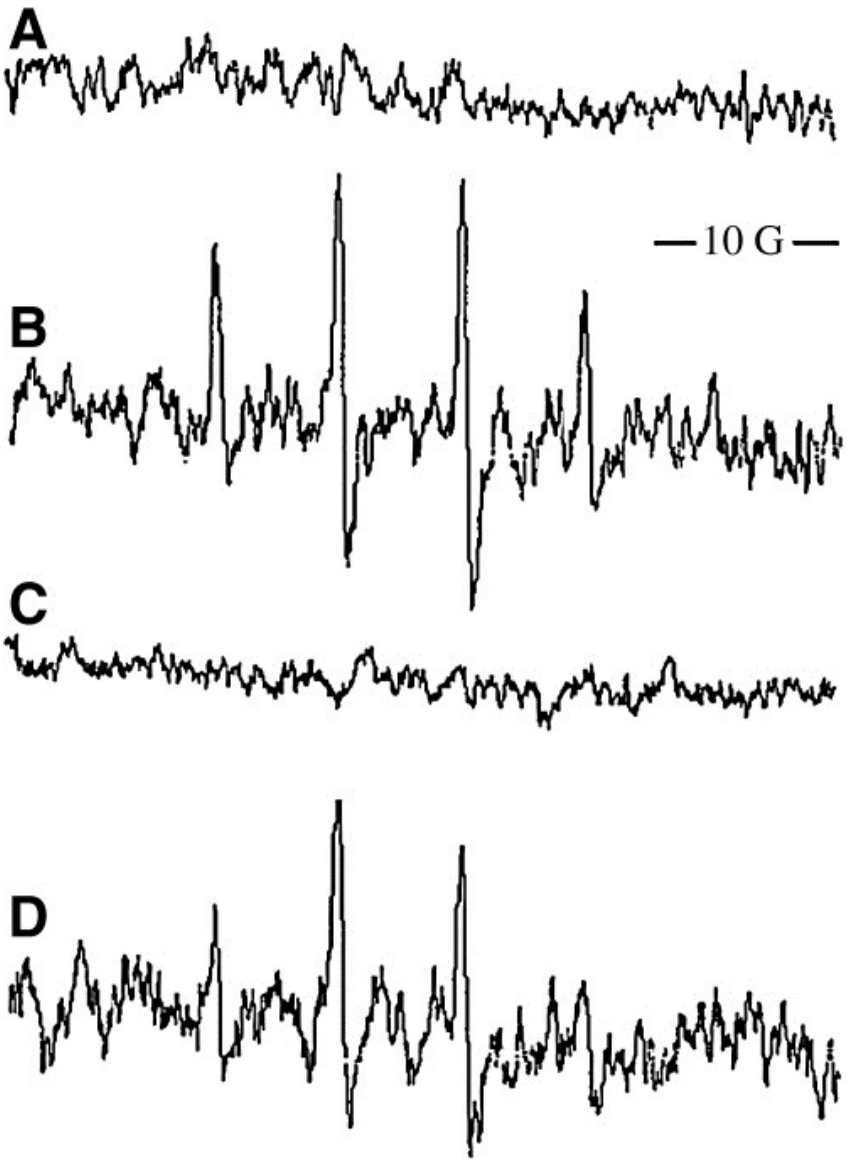

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Fig. 5

Representative traces of H_2O_2 emission rates (amplex red, HRP) during 10 m*M* succinate–supported respiration in guinea pig heart isolated mitochondria. H_2O_2 emission was abrogated (**A**) by adding 4 µ*M* carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), a mitochondrial uncoupler, or by 4 µ*M* rotenone (**B**), a complex I blocker. H_2O_2 emission during pyruvate (complex I substrate, 10 µ*M*)-supported respiration (**C**). Catalase (300 U/ml) was added to scavenge the H_2O_2 generated, and 5 µ*M* antimycin A (**AA**) was added to enhance the H_2O_2 generated at complex III. Note the lower rate of H_2O_2 emission with pyruvate than with succinate (reverse electron transfer). afu, arbitrary fluorescence units. Numbers are changes in afu/min. [Reprinted with permission from Heinen *et al*. (158)].

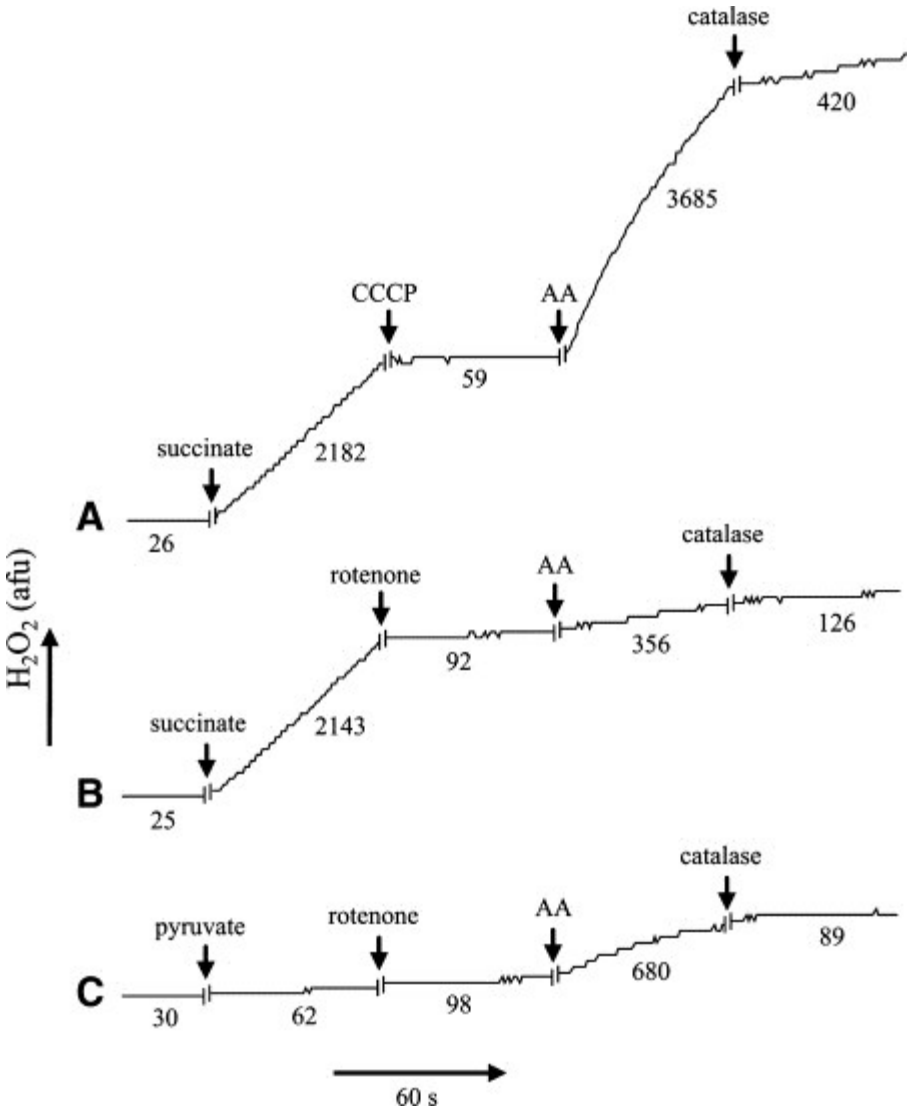

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Fig. 6

DHE (dihydroethidium) fluorescence ($O_2^{\bullet -}$) during perfusion of guinea-pig isolated hearts at 37°C and 17°C with and without four drugs. Cardiac cooling markedly increased $O_2^{\bullet -}$ emission. MnTBAP, a SOD mimetic, reduced $O_2^{\bullet -}$ emission, and menadione (vitamin K_3), an electron-transport inhibitor, increased $O_2^{\bullet -}$ emission, whereas BDM (butanedione monoxime), a contractile inhibitor, and L-NAME (N^G-nitro-l-arginine methyl ester), an inhibitor of $NO^{\bullet}$ synthesis, had no effect on $O_2^{\bullet -}$ emission. DHE is thought to react with $O_2^{\bullet -}$ to form 2-OH-E^+, which produces a transient red spectral shift. [Reprinted with permission from Camara *et al.* (71)].

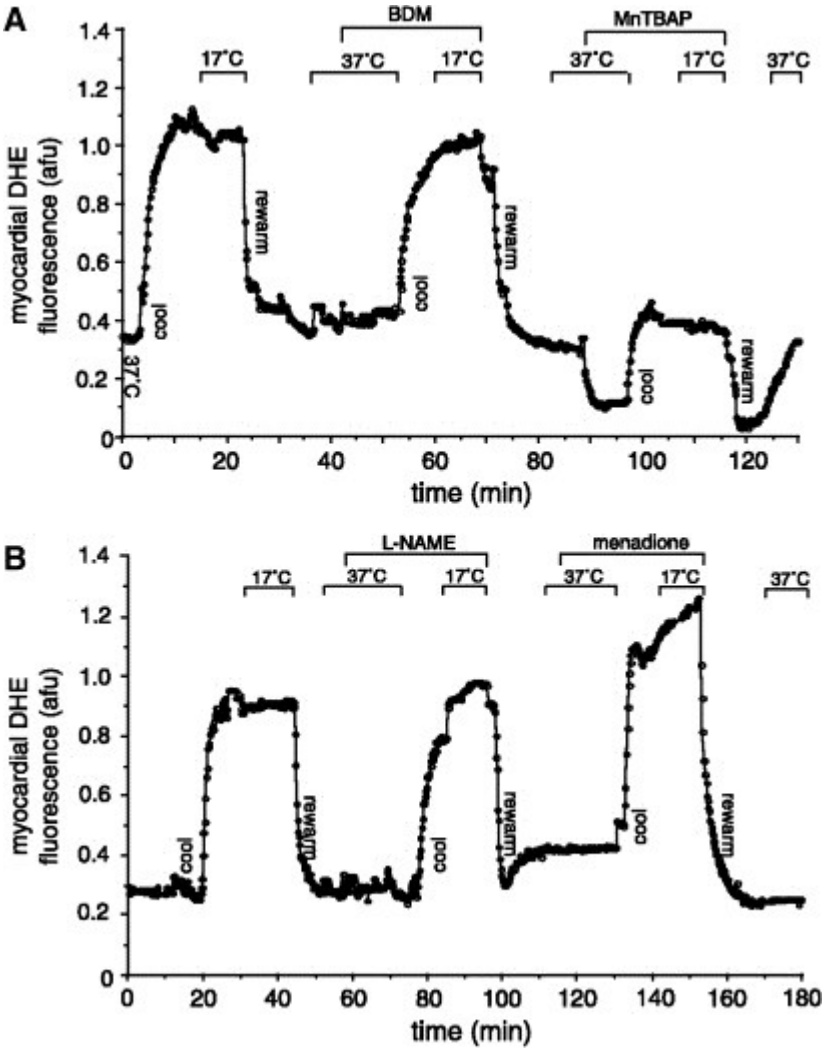

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Fig. 7

Dityrosine (diTyr) fluorescence ($ONOO^-$) during perfusion of guinea-pig isolated hearts at 37°C and 17°C with and without four drugs. Cardiac cooling markedly increased $ONOO^-$. MnTBAP, a SOD mimetic, and L-NAME (N^G-nitro-l-arginine methyl ester), an inhibitor of $NO^\bullet$ synthesis, both reduced $ONOO^-$, whereas menadione (vitamin K_3), an electron-transport inhibitor, increased $ONOO^-$, and BDM (butanedione monoxime), a contractile inhibitor, had no effect on $ONOO^-$. [Reprinted with permission from Camara *et al.* (71)].

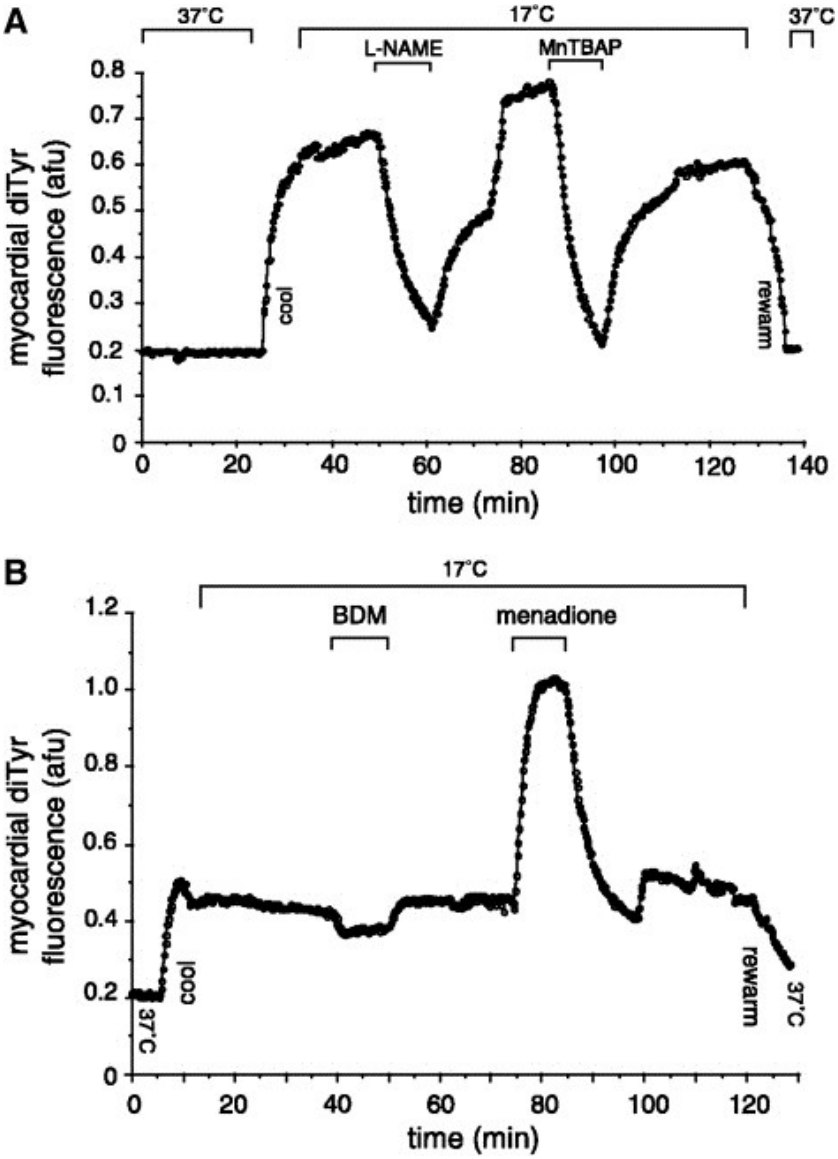

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Table 2.

Drugs Commonly Used to Assess Deductively the Sites of Electron Leak and Superoxide Generation within the Respiratory Chain

Drug	***Mechanism***	***Effect***
Complex I sites	*Inhibitors of NADH oxidation*	
Rotenone (ROT)	Inhibits transfer of e^- from Q-binding site to complex I	+ $O_2^{\bullet -}$
Amobarbital (AMO)	Blocks transfer of e^- from Q_{Nf} to Q_{Ns} distal to N2 Fe/S center	+ $O_2^{\bullet -}$
Piericidin A (PCA)	Inhibits e^- by competing with Q_B for binding site in complex I	+ $O_2^{\bullet -}$
Complex II sites	*Inhibitors of succinate dehydrogenase*	
Malonate (MAL)	Competitive inhibitor of complex II	+ $O_2^{\bullet -}$
Methylmalonate (MML)	Competitive inhibitor of complex II	+ $O_2^{\bullet -}$
3-Nitroproprionate (3NP)	Irreversible inhibitor of complex II	+ $O_2^{\bullet -}$
Atpenins (APT)	Noncompetitive inhibitor of complex II	+ $O_2^{\bullet -}$
Complex III sites	*Inhibitors of quinone (Q) cycle*	
Stigmatellin (STG)	Prevents transfer of $1^{st}e^-$ from QH_2 to Fe/S protein (ISP)	± $O_2^{\bullet -}$[a]
Myxothiazol (MYX)	Prevents binding of QH_2 at Q_o site	+ $O_2^{\bullet -}$
Antimycin A (ANA)	Prevents transfer of $2^{nd}e^-$ to Q_1 site	+ $O_2^{\bullet -}$
Complex IV sites	*Inhibitors of O_2 binding*	
K^+ cyanide (KCN)	Allosterically inhibits cytochrome oxidase	± $O_2^{\bullet -}$[b]
Na^+ azide (NaN_3)	Allosterically inhibits cytochrome oxidase	± $O_2^{\bullet -}$
Carbon monoxide (CO)	Competes with binding of O_2 to cytochrome oxidase	± $O_2^{\bullet -}$
Nitric oxide ($NO^{\bullet}$)	Competes with binding of O_2 to cytochrome oxidase	± $O_2^{\bullet -}$
	General oxidants	

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Drug	***Mechanism***	***Effect***
Menadione ($vitK_3$)	Stimulates redox cycling by one e^- reductive enzymes	$+ O_2^{\bullet -}$
Quinones, doxorubicin	Reacts with Fe^{3+} compounds to produce $Fe^{2+} + e^-$	$+ O_2^{\bullet -}$
H_2O_2	Reacts with Fe compounds to produce $Fe(IV = O)^{\bullet}$ and $Fe^{+3} + {}^{\bullet}OH + {}^{-}OH$ Fenton effect (minor)	$+ {}^{\bullet}OH$

Note: Effects of some drugs on $O_2^{\bullet -}$ generation[a,b] depend on the substrate used and whether other drugs that act on the respiratory system are present.

+, increase; −, decrease.

Fig. 8.

Schema of proposed $O_2^{\bullet -}$ generation sites in complex I at FMN (flavin), Fe-S centers N1–N5, and/or Q binding sites (A–C, *circles*). Electron transport can be forward (solid arrow) or reverse (*dashed arrow*); $O_2^{\bullet -}$ release is into the matrix only. SDH, succinate dehydrogenase. [Used with permission of and modified from Brand *et al.* (48)].

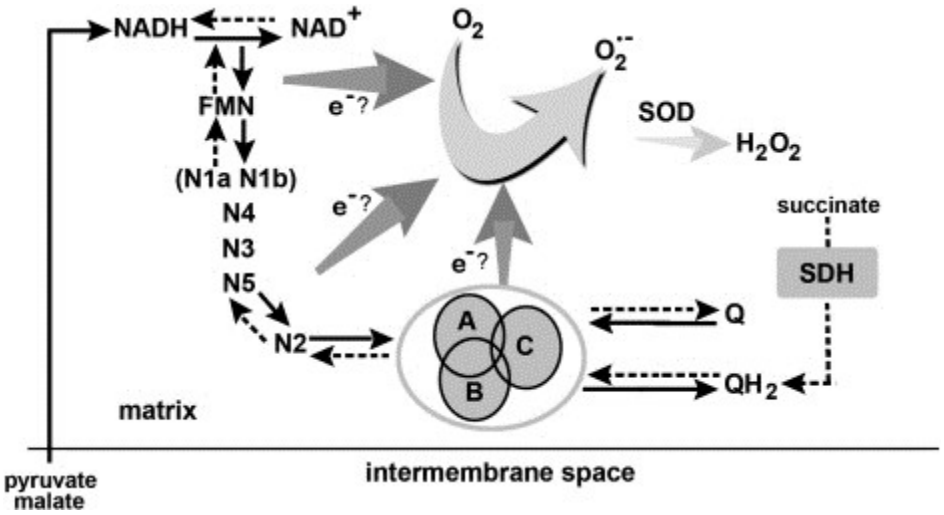

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Fig. 9

Model of $O_2^{\bullet -}$ generation during forward (*top*) and reverse (*bottom*) electron transfer within complex I. During forward transport (substrate pyruvate), e^- are passed from NADH to Q in a quinone-reducing site *via* the FMN and Fe-S centers. The resulting $QH^\bullet$ is reduced in a ΔpH-dependent generating step to form QH_2 with another e^- from QH_2 or $QH^\bullet$ at the quinol oxidation site. $O_2^{\bullet -}$ generation is low unless Q-site inhibitors are present and the ΔpH is large. During reverse transport (substrate succinate), e^- are passed from QH_2 to NAD, so that the pool is reduced to NADH. A large ΔpH drives the formation of $QH^\bullet$, which loses its unpaired e^- to O_2 because all redox centers upstream of Q are fully reduced. [Used with permission of and modified from Lambert and Brand (200)].

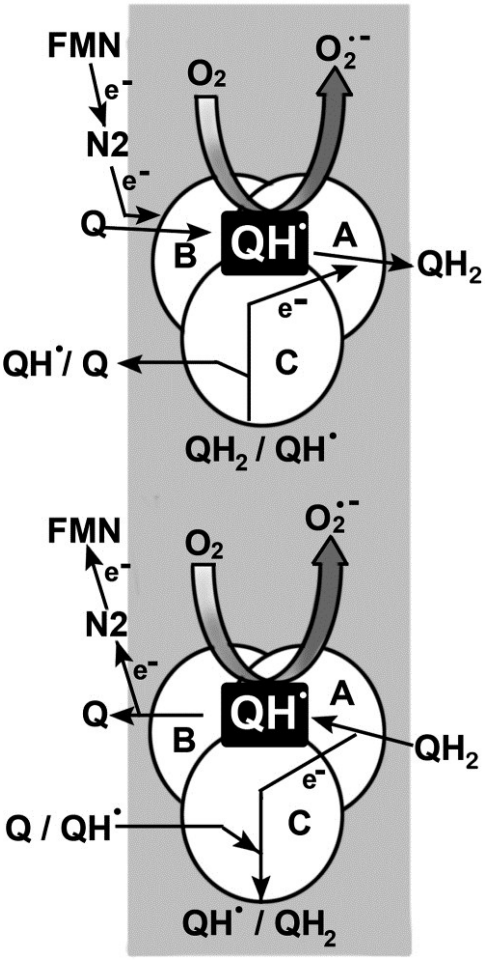

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Fig. 10

H_2O_2 production is more dependent on mitochondrial ΔpH than on $\Delta\Psi_m$ during reverse electron transfer at complex I. This is suggested by the dependence of H_2O_2 production on $\Delta\Psi_m$ when ΔpH is present (**a**) or absent (**b**); the difference (**c**) represents H_2O_2 production as a function of ΔpH. ΔpH was abolished with nigericin (**b**), which converts any ΔpH into $\Delta\Psi_m$. [Reprinted with permission from Lambert and Brand (201)].

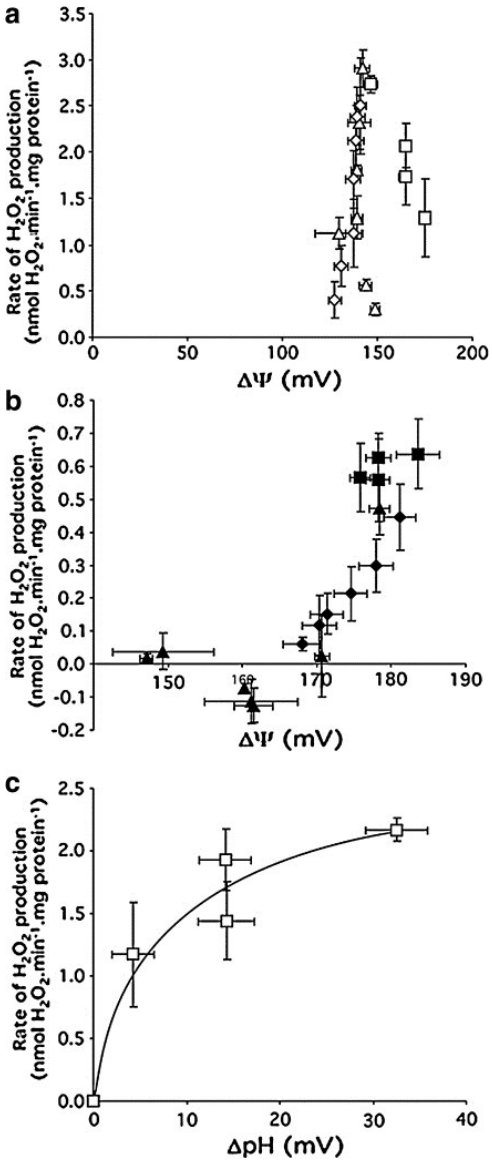

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Fig. 11

Mechanisms of $O_2^{\bullet -}$ formation from complex III. Oxidation of quinol (QH_2) results in transfer of a single e^- to a high-reduction-potential chain at the Q_o (extramatrix) site containing the Fe-S protein (ISP, Rieske protein) and on to cytochrome c_1 and cytochrome *c*, and finally to cytochrome *c* oxidase. The remaining $QH^{\bullet}$ is unstable and donates the second e^- to a low-reduction-potential chain consisting of cytochrome b_l (low) and b_h (high), leading the e^- toward the Q_i (matrix) site to form a stable $QH^{\bullet}$; this $QH^{\bullet}$ is then reduced to QH_2 by a subsequent e^- passed along the low-reduction-potential chain, thereby completing the Q–QH_2–Q cycle. In (**A**), antimycin A causes extramatrix release of $O_2^{\bullet -}$; in (**B**), stigmatellin and myxothiazol block antimycin A from releasing $O_2^{\bullet -}$. [Used with permission of and modified from Andreyev *et al*. (8)].

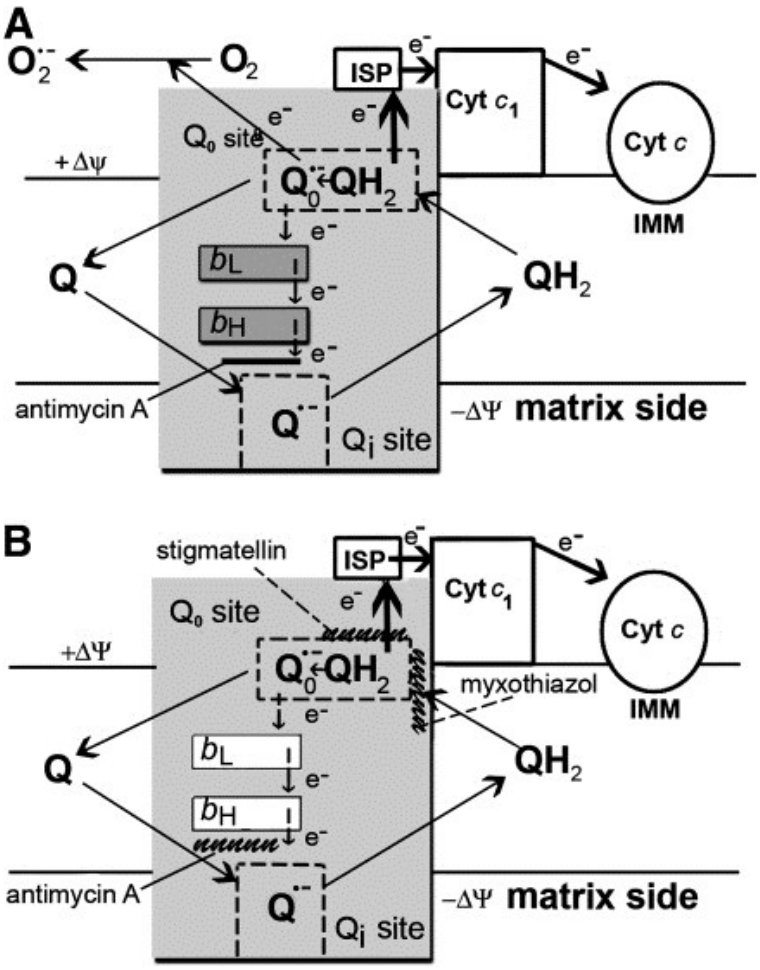

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Fig. 12

Highly reduced and coupled mitochondria (high $\Delta\Psi_m$ and limited respiration due to lack of ADP) leak e^- for attack by O_2 because $QH^\bullet$ is not as rapidly reduced to QH_2. Progressive uncoupling by using a protonophore (SF6847) gradually reduces $\Delta\Psi_m$ and increases respiration while markedly preventing H_2O_2 generation. A similar effect occurs during state 3 with added ADP. [Reprinted with permission from Korshunov *et al.* (191)].

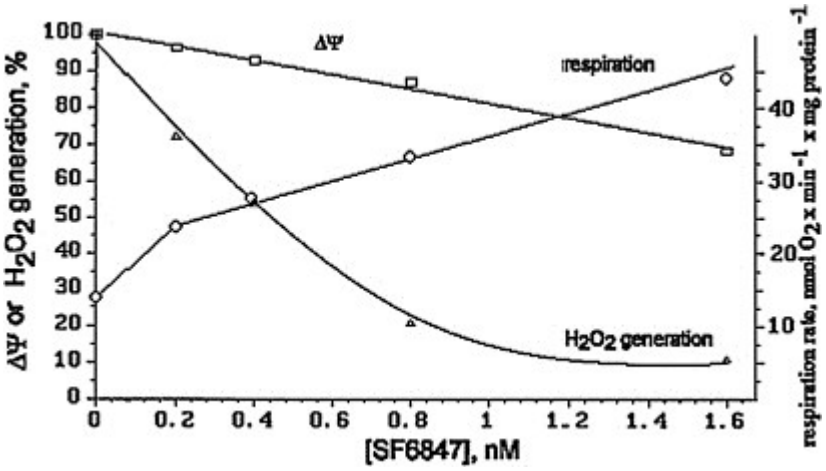

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Fig. 13

Isolated mitochondria can produce H_2O_2 during state 3 (ADP stimulated) as well as during state 4 conditions during forward electron flow, provided that [O_2] and mitochondrial protein concentration are sufficient. [Reprinted with permission from Saborido *et al.* (276)].

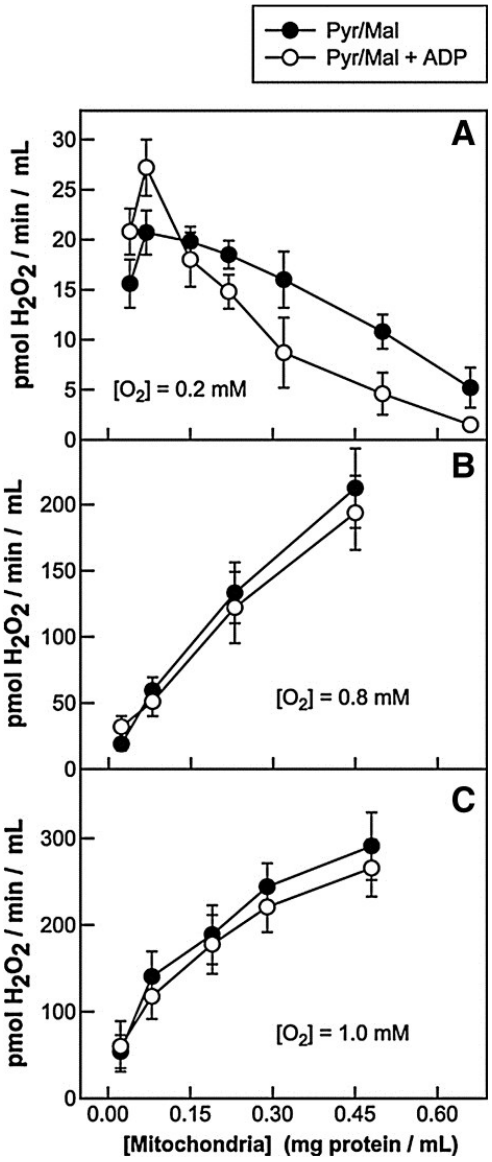

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Fig. 14

ROS production at complex I in cardiac mitochondria is critically dependent on a highly reduced NADH pool. Downstream inhibition of electron transfer (respiration) by rotenone leads to reduction of all upstream carriers and results in e^- leak and ROS generation. Note the large decrease in respiration rate and the increasingly reduced redox state with rotenone associated with ROS production during state 3 (*left*) compared with state 4 (*right*). [Reprinted with permission from Kushnareva *et al.* (199)].

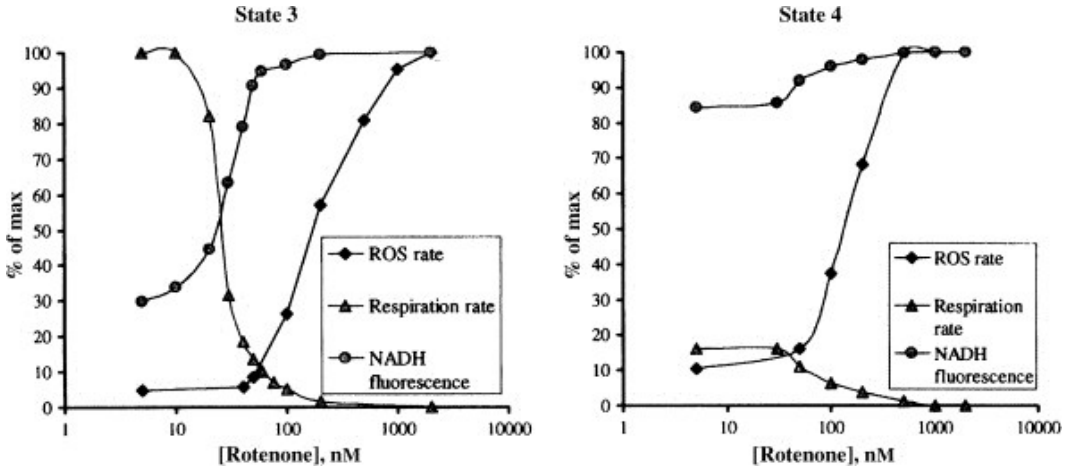

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Fig. 15

DHE (dihydroethidium) fluorescence ($O_2^{\bullet -}$ emission) increases during perfusion of guinea-pig isolated hearts during intermittent (A) and continuous (B) cooling from 37°C to 2°C. The increase in $O_2^{\bullet -}$ emission with cooling likely results both from increased $O_2^{\bullet -}$ generation and decreased $O_2^{\bullet -}$-scavenging capability by MnSOD. Afu, arbitrary fluorescence units. [Reprinted with permission from Camara *et al.* (71)].

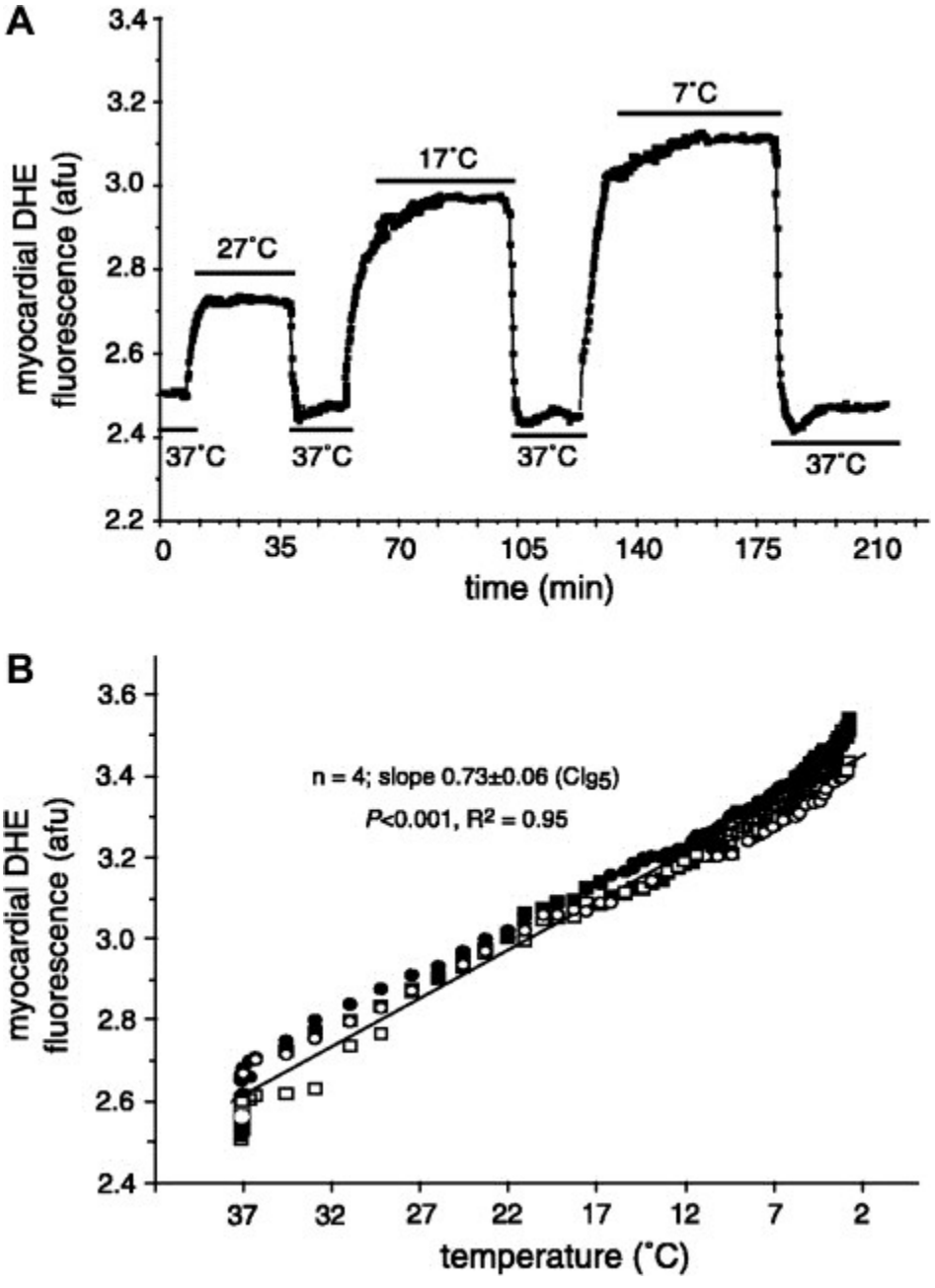

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Fig. 16

Temporal relation between $\Delta\Psi_m$ (TMRM fluorescence) and ROS (DCF fluorescence) in a cardiac myocyte. "Trigger" ROS was induced by photoactivation of TMRM derivatives. Note the burst of ROS after photoexcitation by laser scanning caused a decrease in $\Delta\Psi_m$, which was assumed to be a result of ROS-induced MPT opening. [Reprinted with permission from Zorov *et al.* (336)].

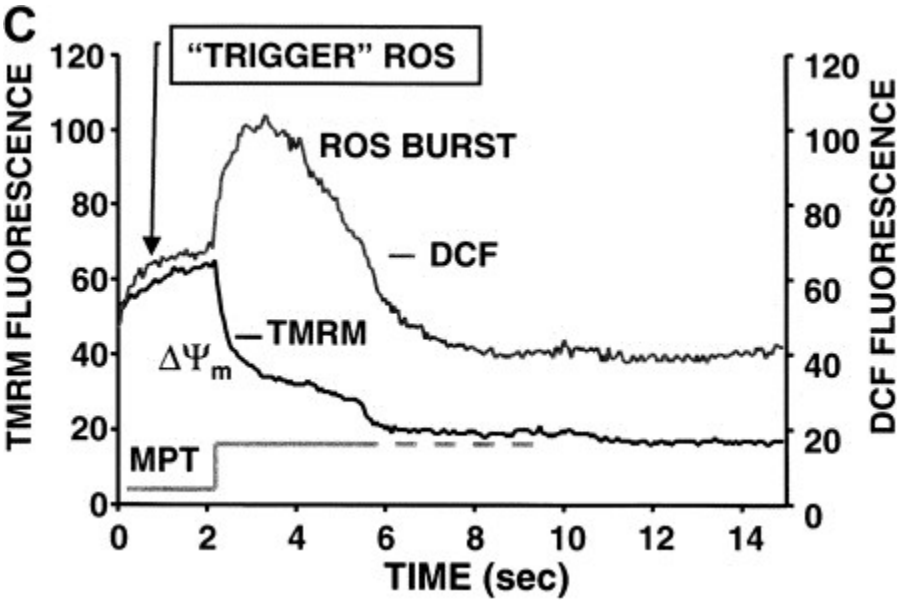

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Fig. 17

Averaged mitochondrial [Ca^{2+}] (A), NADH (B), and DHE ($O_2^{\bullet -}$) (C) in four groups of guinea-pig isolated hearts over time. Groups are time control *versus* 30-min global ischemia (isc) at 37°C *versus* 17°C. Note the increases in mitochondrial [Ca^{2+}] and $O_2^{\bullet -}$, while NADH decreases, during later ischemia. Variables were measured in the left ventricle with a trifurcated fiberoptic probe and differential fluorescence spectrophotometry. [Reprinted with permission from Riess *et al.* (269)].

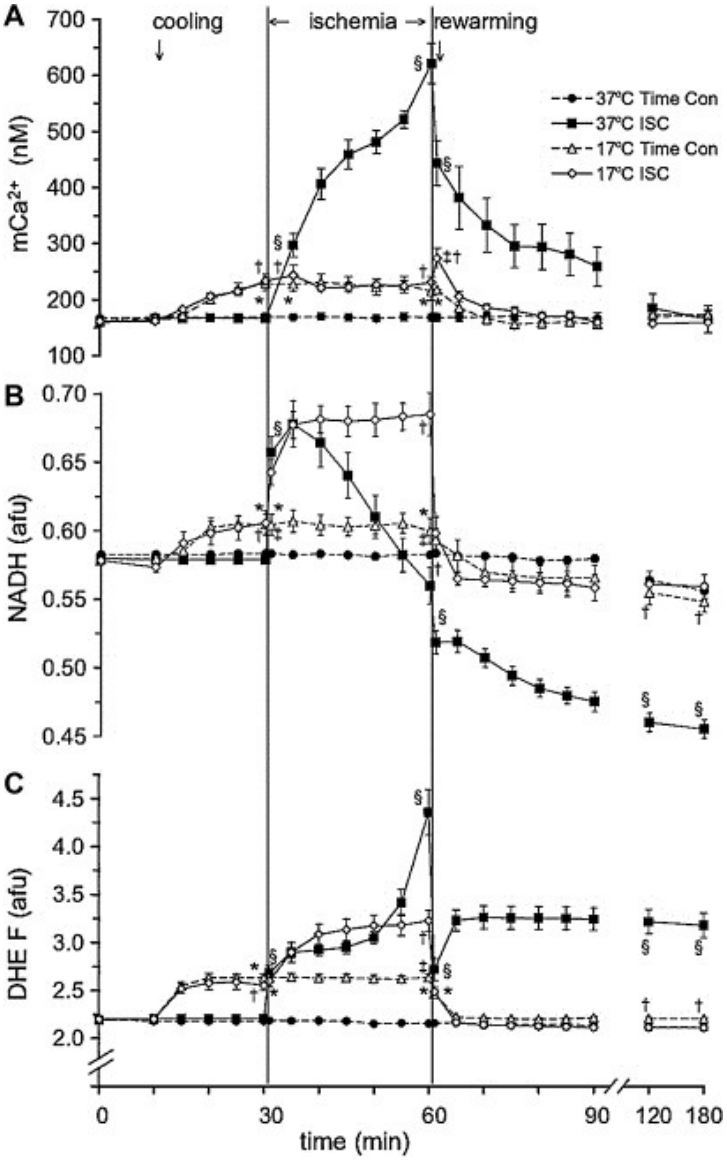

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Fig. 18

Averaged DHE ($O_2^{\bullet -}$) (A) and mitochondrial [Ca^{2+}] (B), in five groups of guinea-pig isolated hearts over time. Groups are 30-min global ischemia control and preischemia treatments with MnTBAP, catalase + glutathione (CG), MnTBAP + CG (MCG), and l-NAME. Note that the MnTBAP-treated group exhibited the largest increase in [Ca^{2+}], whereas the MCG-treated group exhibited the smallest increases in [Ca^{2+}] and $O_2^{\bullet -}$. [Reprinted with permission from Camara *et al.* (70)].

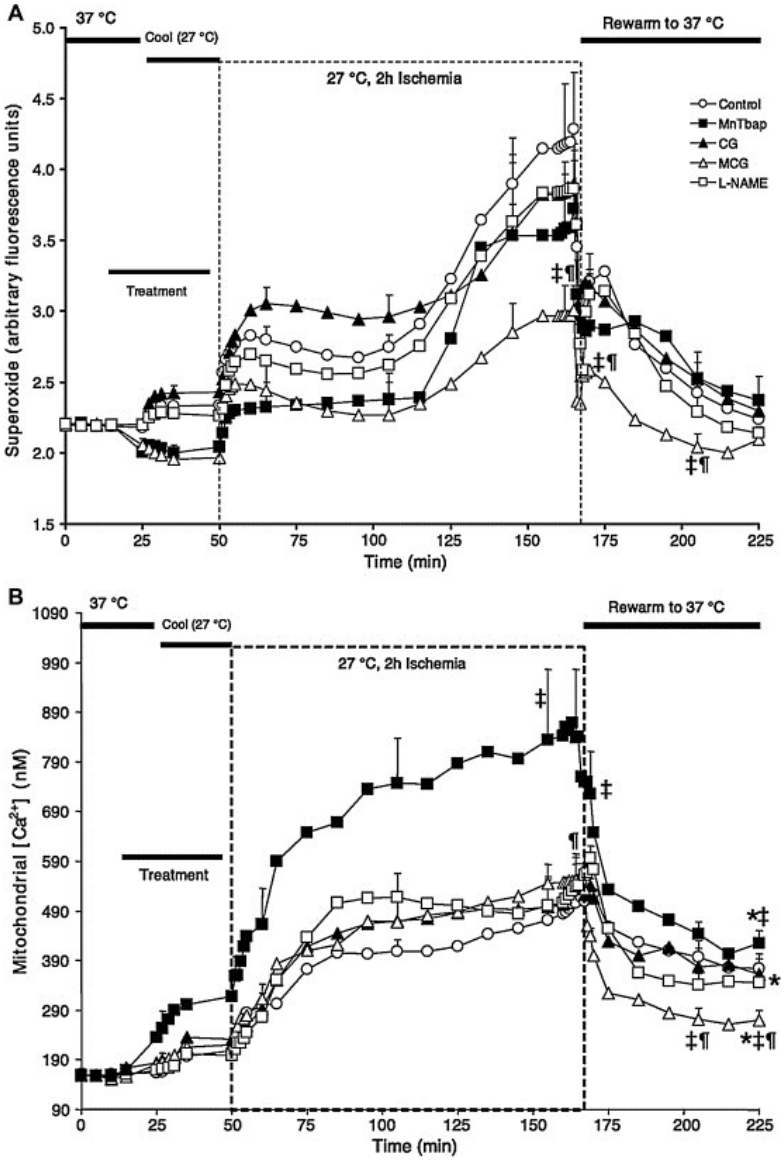

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Fig. 19

Averaged ETH (DHE) fluorescence ($O_2^{\bullet -}$) in four groups of guinea-pig isolated hearts over time. Note that the increase in $O_2^{\bullet -}$ during brief global ischemia (IPC, ischemic preconditioning) was attenuated by MnTBAP and that MnTBAP also blocked the reduction in $O_2^{\bullet -}$ afforded by IPC so that $O_2^{\bullet -}$ increased to the level of the ischemia control. [Reprinted with permission from the publisher of Kevin *et al.* (181)].

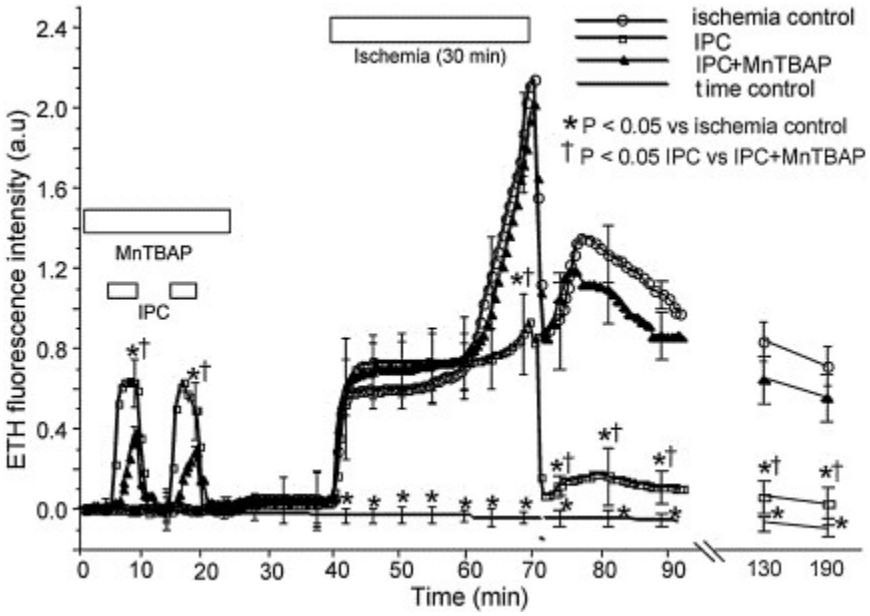

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Fig. 20

Dependence of H_2O_2 production during state 3 (A) and state 4 (B) on $[O_2]$ in mitochondria isolated from rat liver. Note that at <5 μ*M* $[O_2]$, H_2O_2 production decreased toward zero. Experiments were conducted in the presence of succinate + rotenone and oligomycin (state 4 only). [Reprinted with permission from Hoffman *et al.* (161)].

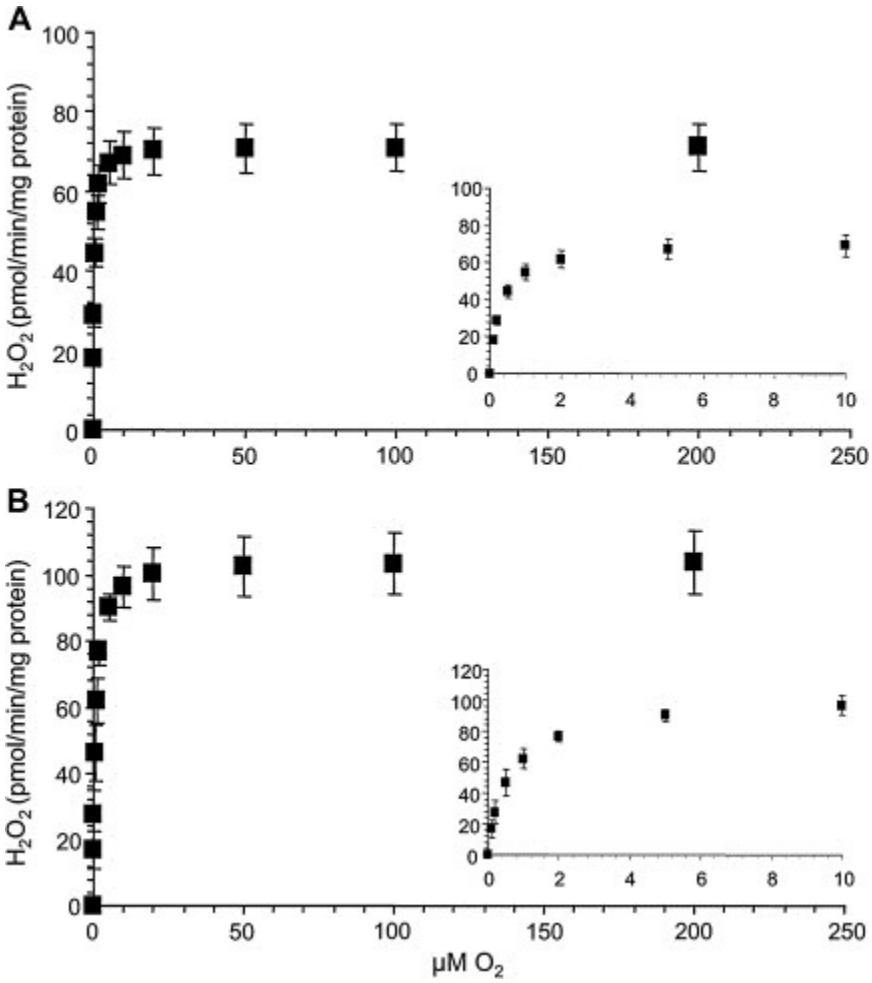

Fig. 21

Schema of glutaredoxin and thioredoxin buffering system in the mitochondrion. Glutathione is transported across the outer membrane (OM) *via* porin into the intermembrane space (IMS) and across the inner membrane (IM) *via* a transporter (trans) to the matrix. Other redox proteins are transported *via* the TOM and TIM23 complexes. The glutaredoxin and thioredoxin reactions in the matrix repair oxidatively damaged proteins. [Used with permission and modified from Koehler *et al*. (189)].

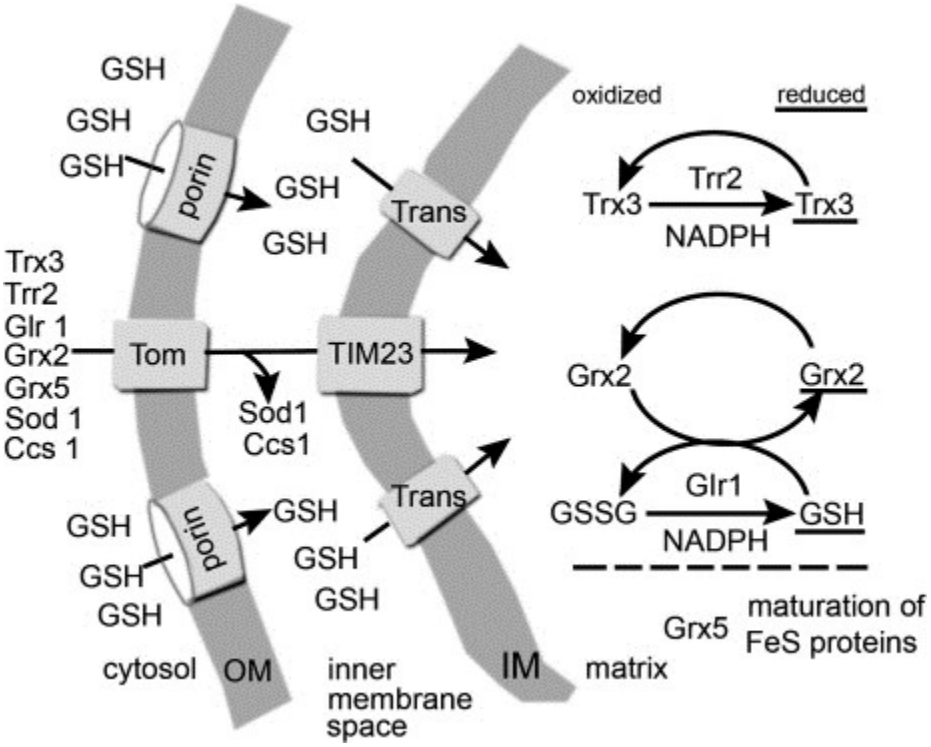

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Fig. 22

Pathways of reactive O_2 metabolism. $O_2^{\bullet -}$ and H_2O_2 exert protective effects on the cell *via* signaling pathways for preconditioning *via* phosphorylation products and *via* oxidant-induced gene products that activate multiple groups of proteins. Severe hypoxia, ischemia and reperfusion, and toxins can cause excessive oxidant stress that leads to cell-damaging effects of ferryl radicals such as Fe(IV) = O•. [Used with permission and modified from Becker (27)].

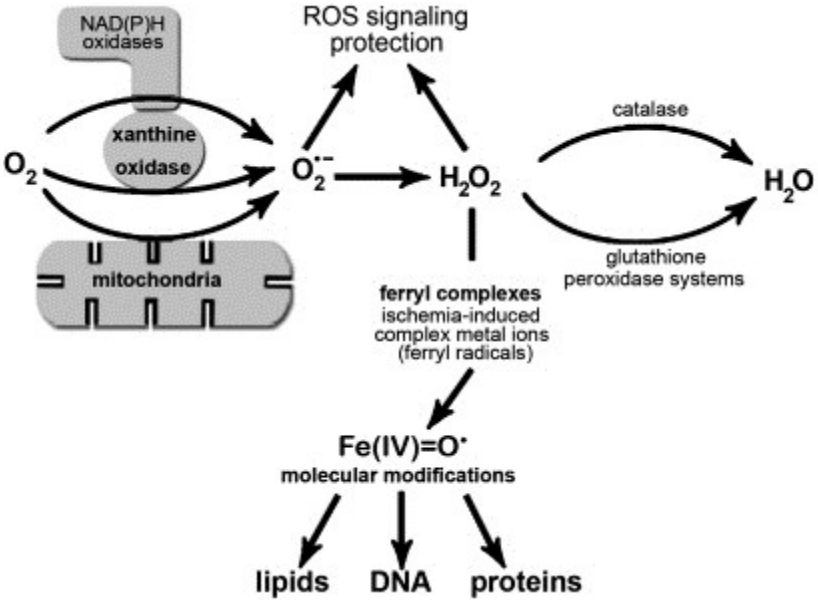

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Fig. 23

Pathways of NO•, $O_2^{\bullet -}$, and HO• and their reactive lipid products, including lipid peroxides (1), 15-deoxy-$\Delta^{12,14}$-protaglandin J_2 (2), isoprostane J_2 (3), 4-hydroxynonenal (4-HNE) (4), acrolein (5), nitrolinoleic acid (6), and lysoPC (7). [Used with permission and modified from Zmijewski *et al.* (334)].

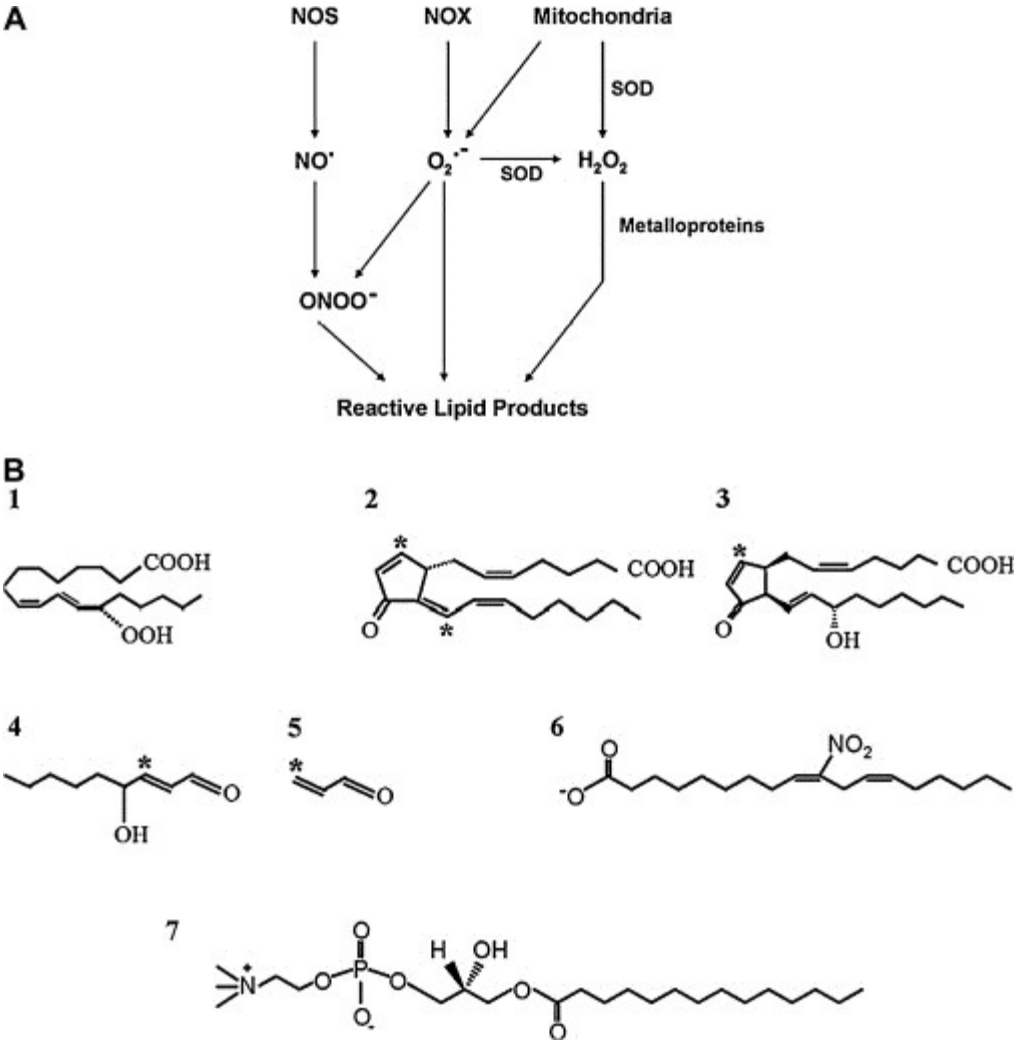

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Fig. 24

Correlations between mitochondrial H^+ leak, metabolic rate, and membrane fatty acid polyunsaturation in liver mitochondria. [Reprinted with permission from Brookes (55)].

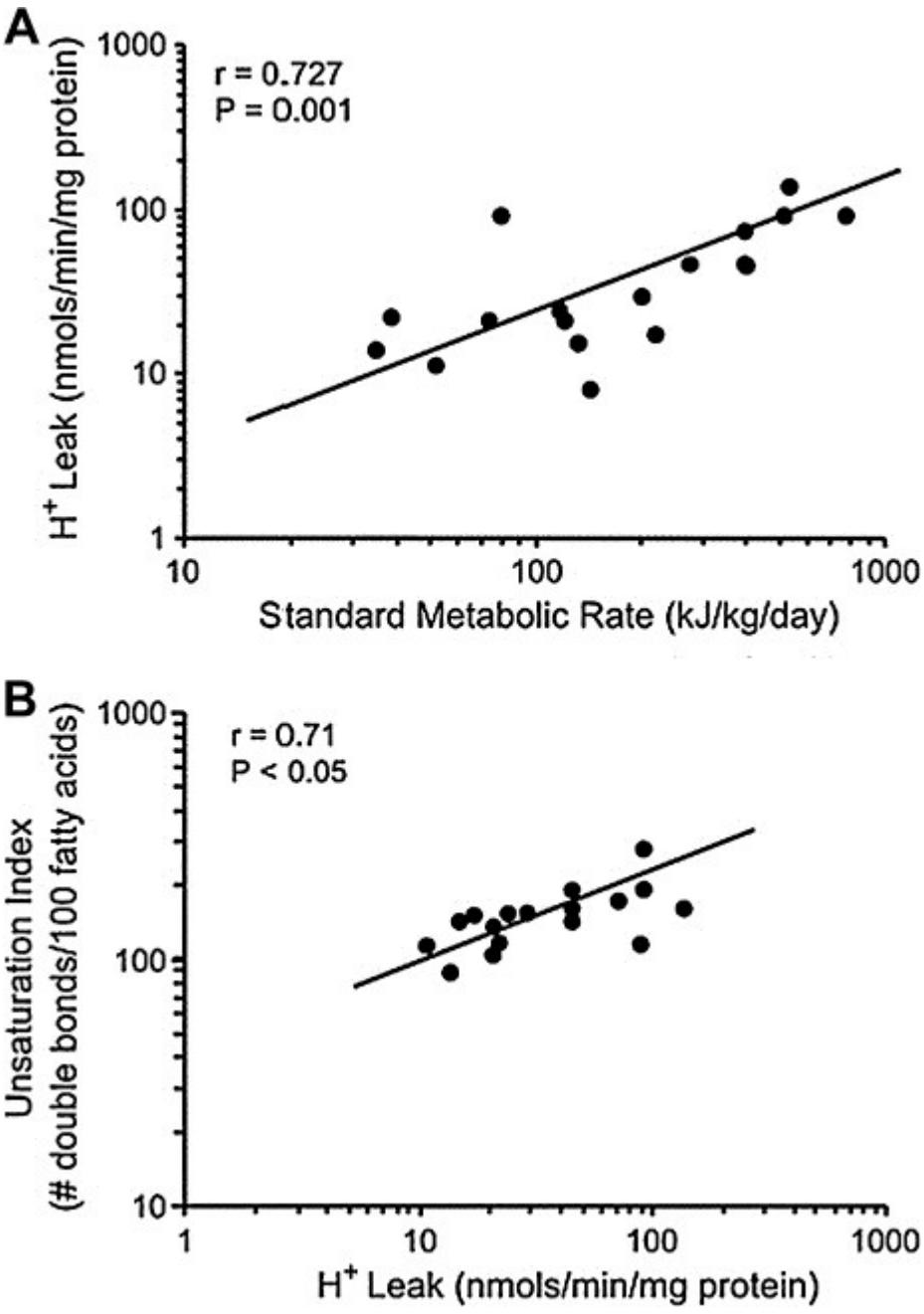

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Fig. 25

Assessment of proton leak from the downward shift in the $\Delta\Psi_m$ *versus* respiratory-rate curve after IPC + ischemia *versus* ischemia alone (IR); no ischemia is control (CON). Note the decrease in respiration after IPC compared with IR at 160 mV (**insert**), indicating less proton leak. Data from rat mitochondria isolated at 30 min of reperfusion after ischemia. [Reprinted with permission from Nadtochiy *et al.* (238)].

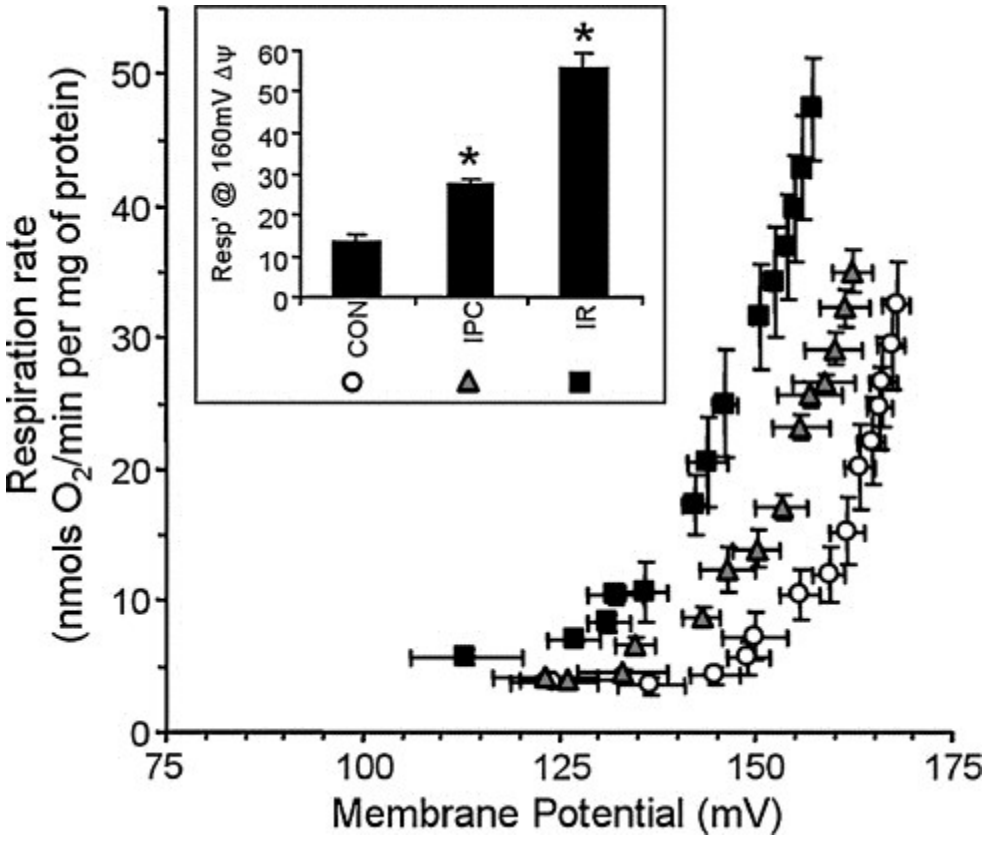

Fig. 26

The K^+ cycle in heart mitochondria. In this model, electrophoretic matrix K^+ influx (leak) is matched by electrogenic H^+ efflux (ETS), and K^+ influx *via* K_{ATP} and K_{ca} channels is matched by K^+ efflux and H^+ influx *via* KHE (K^+/H^+). The H^+ influx is accompanied by phosphate influx (P_i); a net uptake of phosphoric acid and salt occurs, so that matrix swelling occurs. Finally, matrix alkalinization releases the KHE from allosteric inhibition by protons, and its activity increases to match K^+ influx. [Used with permission and modified from Costa *et al.* (95)].

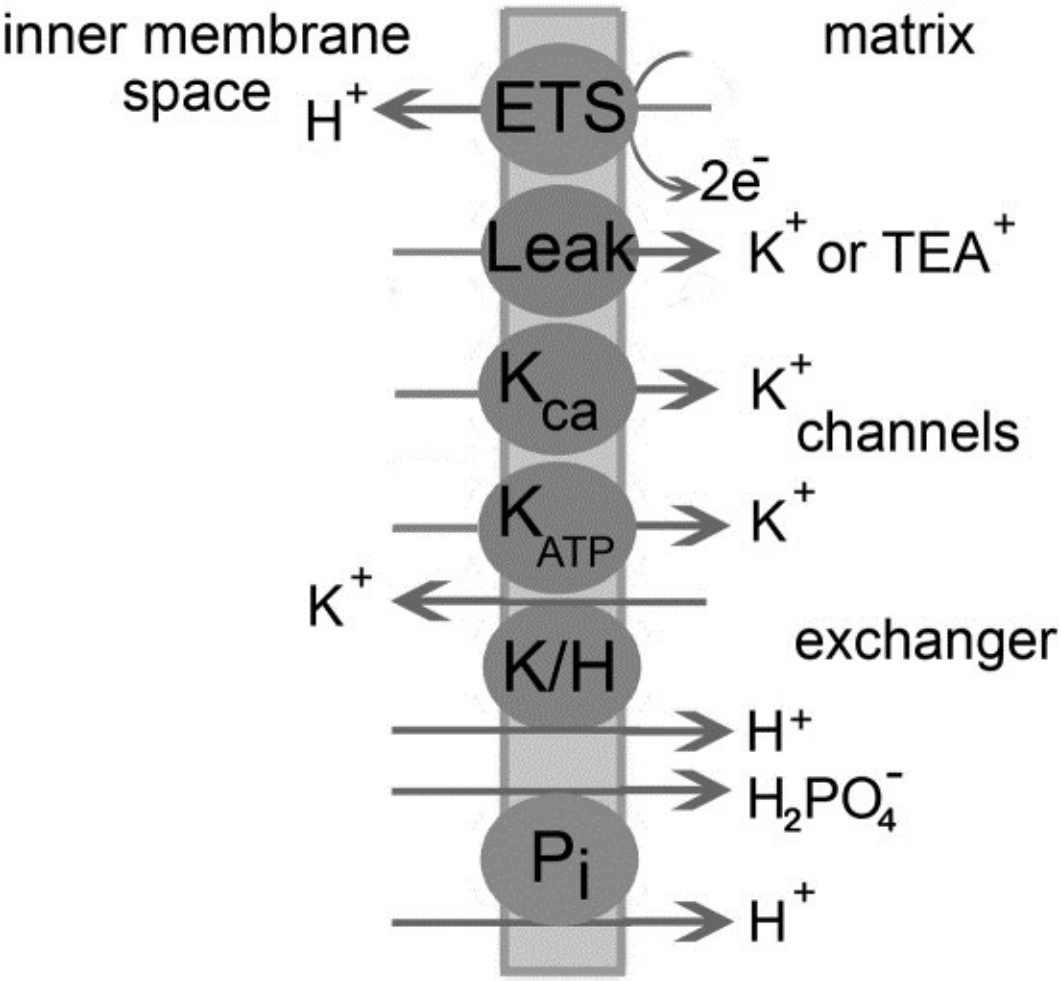

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Fig. 27

Small increases in $O_2^{\bullet -}$ generation assessed by DHE fluorescence (ETH) in isolated hearts during treatment with the anesthetic sevoflurane (Sevo) (A) were blocked by the SOD mimetic MnTBAP (C, D) but not by the K_{ATP} channel inhibitor 5-HD (B, D). [Reprinted with permission from Kevin *et al*. (182)].

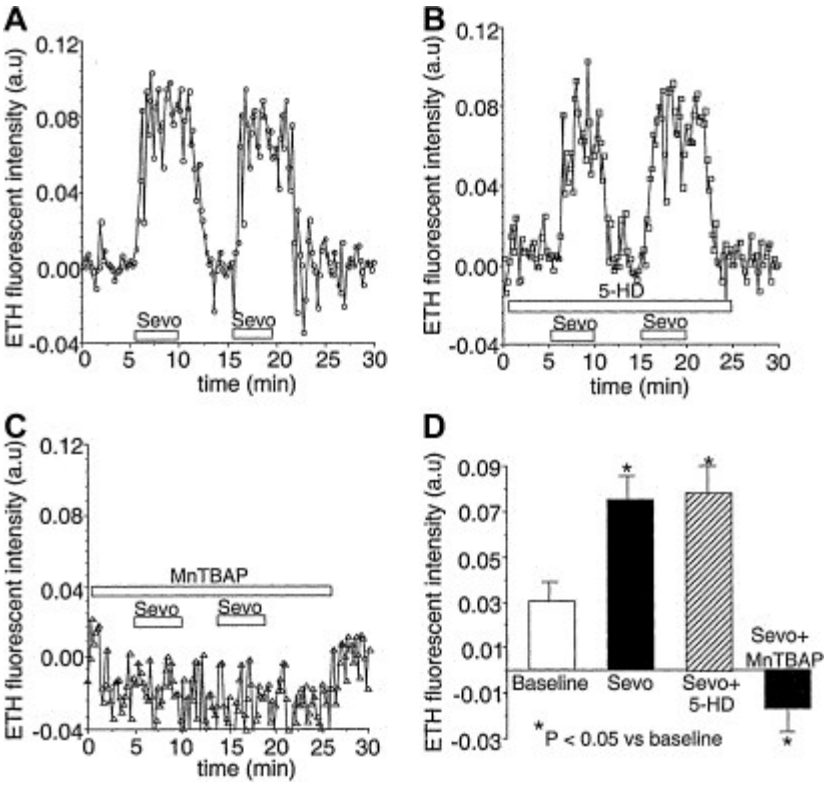

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Fig. 28

Proposed effect of submaximal K^+ influx with mBK_{Ca} channel opening (1) on proton leak (2), proton ejection and respiration (3), $\Delta\Psi_m$ (4) and generation of $O_2^{\bullet-}$ and H_2O_2 (5). Net effect of mBK_{Ca} channel opening (**B**) (vs. closed, **A**) would be to accelerate electron flux without a change in $\Delta\Psi_m$ due to support by proton leak; maintained $\Delta\Psi_m$ and higher electron flow would accelerate ROS generation. [Reprinted with permission from Heinen *et al.* (159)].

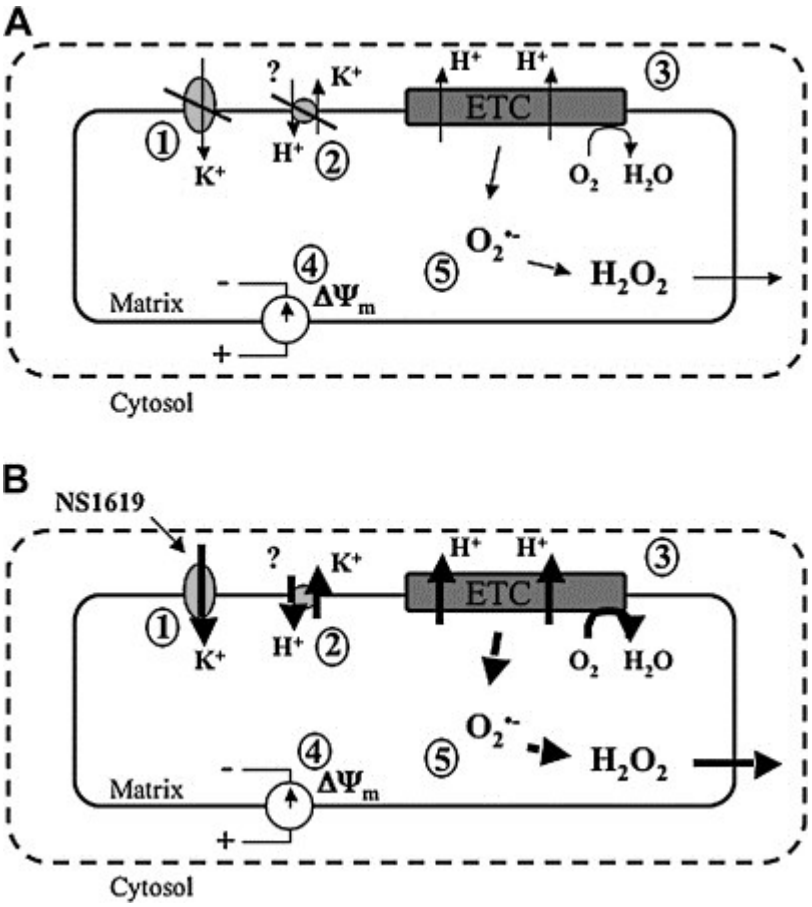

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Fig. 29

Mitochondrial H_2O_2 release rate from heart isolated mitochondria. (**A**) Representative trace for 30 μ*M* NS-1619–induced increase in cumulative H_2O_2 release with succinate + rotenone as substrate. Maximal ROS production was stimulated in some experiments by adding complex III blocker antimycin A (5 μ*M*). Catalase (300 U/ml) was added to confirm H_2O_2 production. *Open arrow a*, baseline; *open arrow b*, treatment effect. (**B**) Summary of H_2O_2 release rates. All treatment effects are compared with baseline of the same experiment. [Reprinted with permission from Heinen *et al.* (159)].

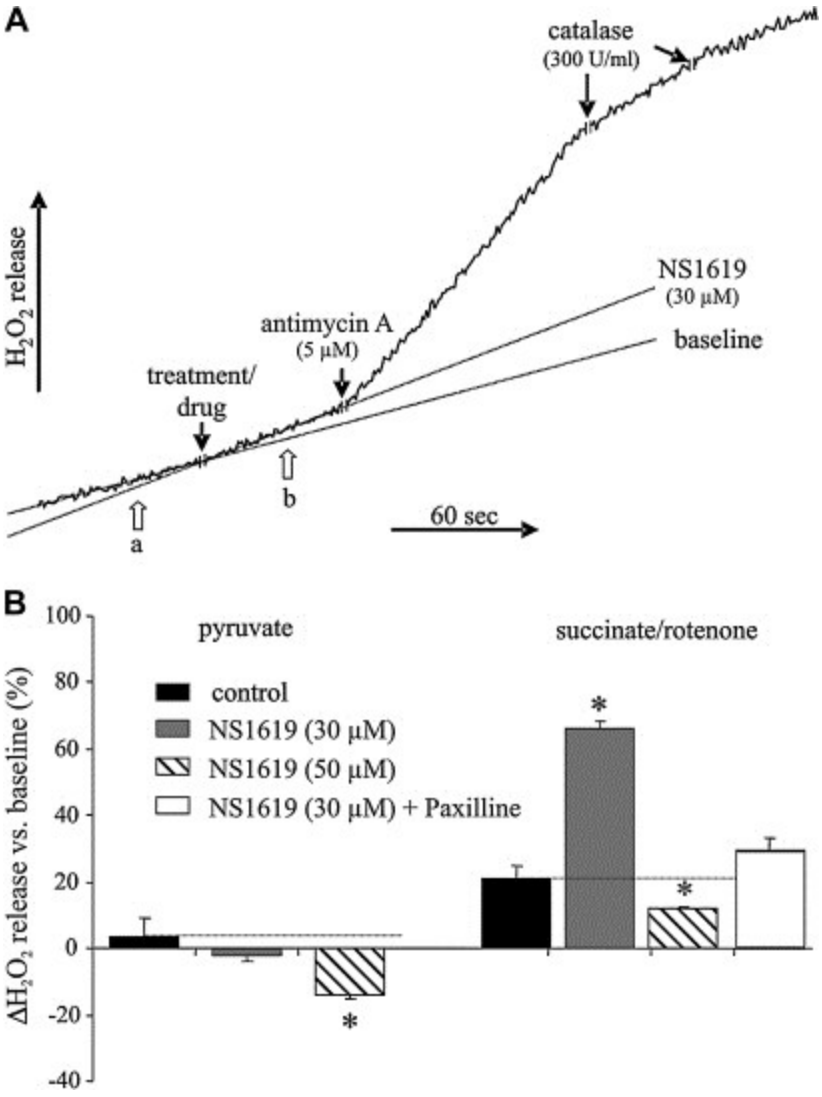

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Fig. 30

ROS production (Cbx-DCF fluorescence) in rat-heart mitochondria over time after adding (before time zero) valinomycin (Val), diazoxide (Dzy), and 5-hydroxydecanoate (5-HD) singly or together with ATP to maintain state 4 (A). ROS production was obtained from the initial slopes of traces such as those shown in (**A**) and plotted as percentage ATP-inhibited control rate obtained in the absence of drug. Adding ROS scavenger *N*-(2-mercaptopropyonyl)glycine (MPG) decreased ROS production in the presence of Dzy and Val ~10-fold (**B**). [Reprinted with permission from Andrukhiv *et al*. (9)].

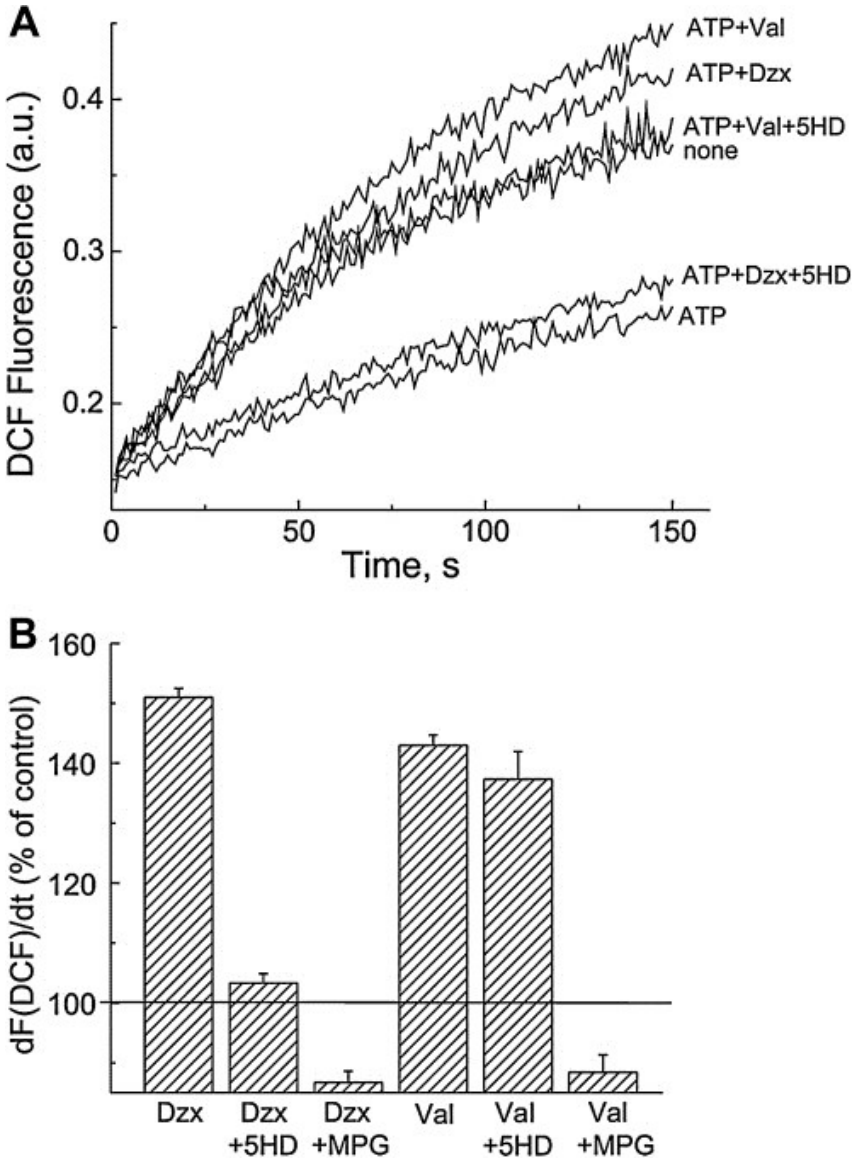

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Fig. 31

Effects of BK_{Ca} channel opener NS1619 on attenuating $O_2^{\bullet -}$ emission (A) and NADH (B) during ischemia and reperfusion in guinea-pig isolated hearts were largely reversed by BK_{Ca} channel blocker paxilline (PX) or by SOD mimetic MnTBAP (TB). *$p < 0.05$ *vs*. Con, NS + PX, NS + TB. [Reprinted with permission from Stowe *et al*. (298)].

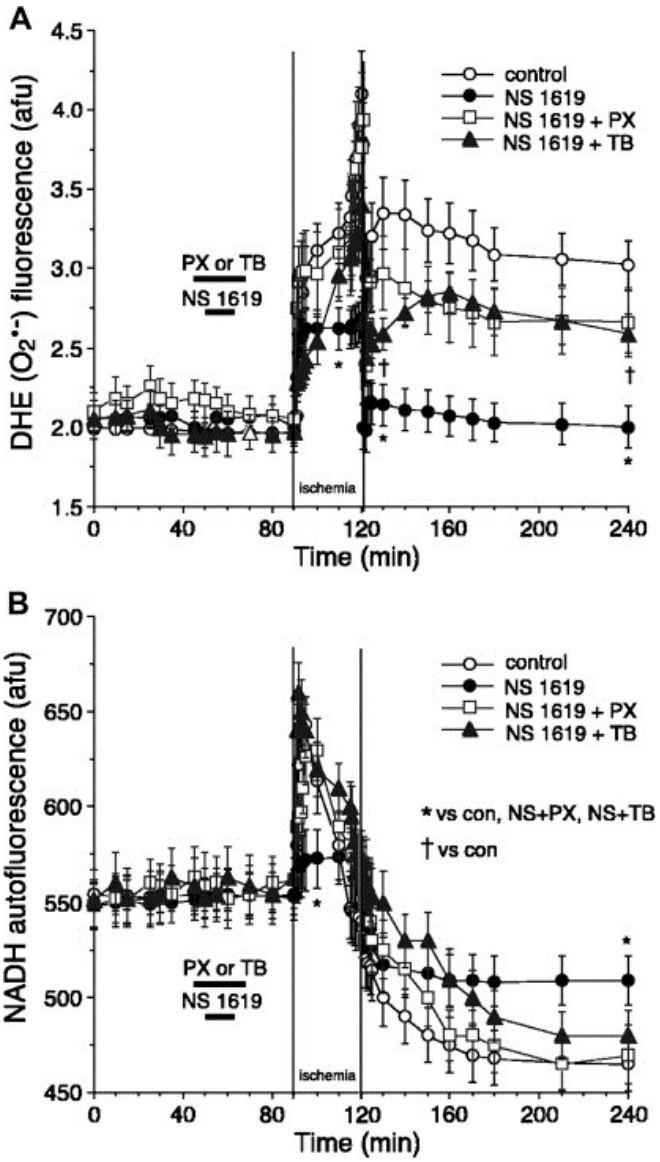

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Fig. 32

Effect of reversible complex I inhibitor amobarbital on $O_2^{\bullet-}$ generation (DHE fluorescence) (A) and mitochondrial [Ca^{2+}] (B) during ischemia and reperfusion in guinea-pig isolated hearts. *Arrow*, 1-min amobarbital perfusion immediately before ischemia. **Inset:** Effect of amobarbital before ischemia on $O_2^{\bullet-}$ emission and [Ca^{2+}]. *White and hatched bars*, baseline and 1 min of treatment, respectively. [Reprinted with permission from Aldakkak *et al.* (5)].

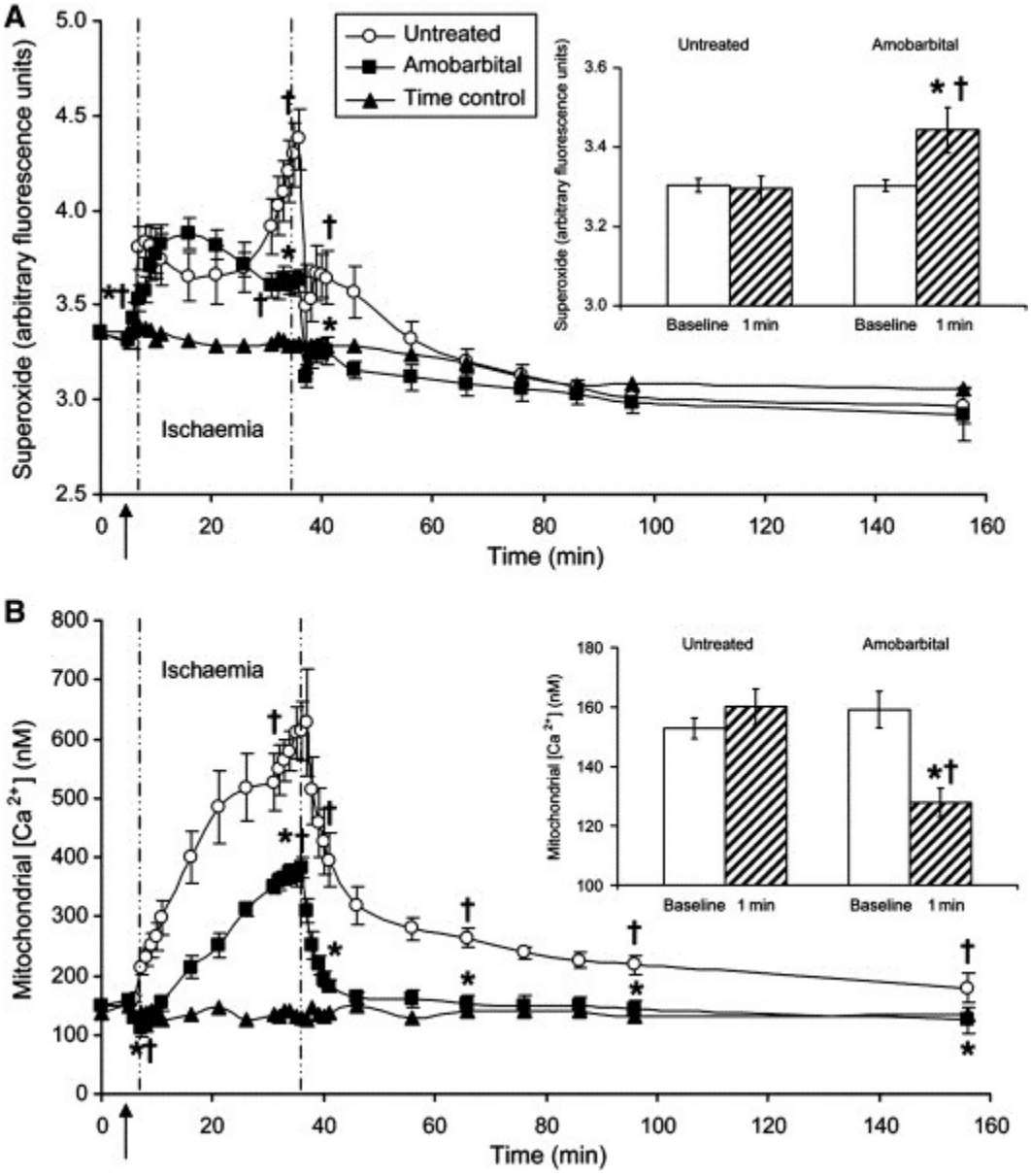

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Fig. 33

Electrons derived from reducing equivalents are transferred through the respiratory system to complex IV where $4e^-$ are transferred to O_2. During hypoxia, this transfer is slowed, even though sufficient O_2 exists to function as the electron acceptor. The resulting buildup of electrons and NADH may set up a condition for electron leak to O_2, partially bypassing complex IV. [Used with permission of and modified from Chandel and Schumacker (80)].

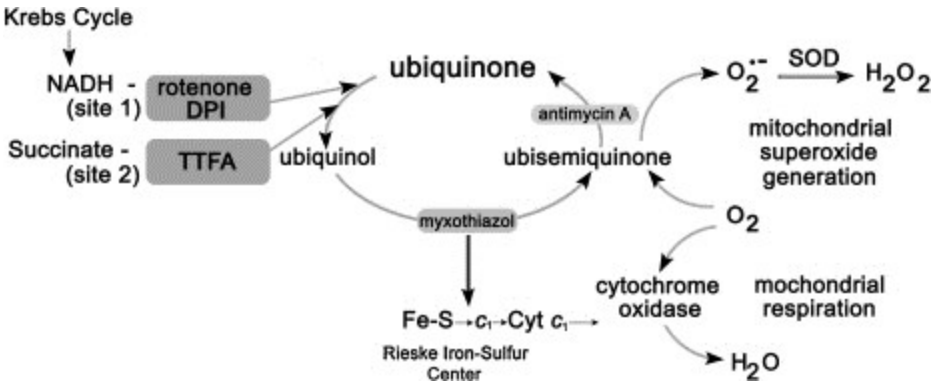

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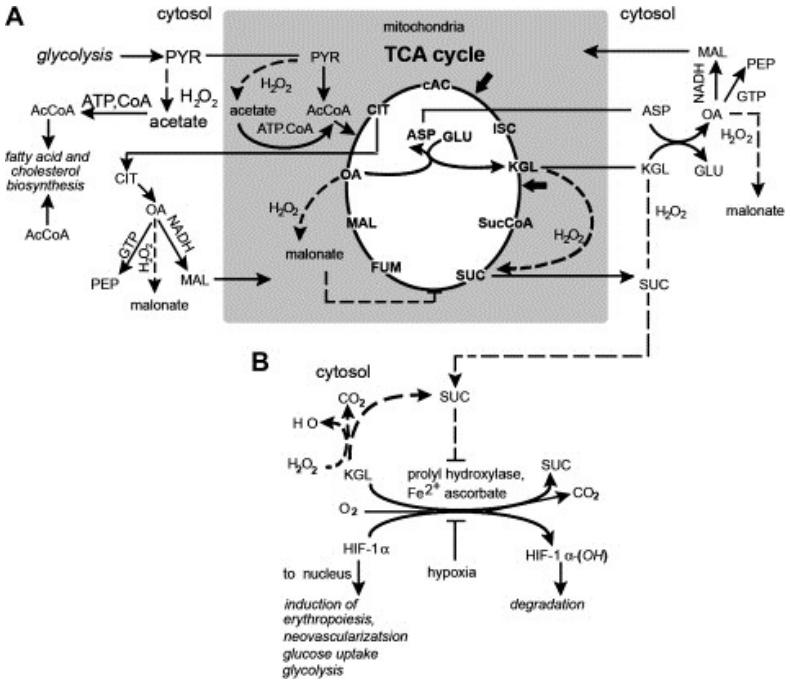
Fig. 34

Effect of H_2O_2 on TCA cycle and products of 2-oxo acid nonenzymatic oxidation on metabolism (A) and signaling (B). (**A**) Pyruvate (PYR), α-ketoglutarate (KGL), and oxaloacetate (OA) are decarboxylated by H_2O_2 to form acetate, succinate, and malonate (*broken line*) nonenzymatically instead of acetyl-CoA (acCoA), succinyl-CoA (sucCoA), and malate (MAL), or phosphoenolpyruvate (PEP) formed enzymatically (*solid line*) and are used to synthesize fatty acids, cholesterol, and glucose. ROS inhibit the TCA cycle mainly at aconitase and α-ketoglutarate dehydrogenase (KGDH) (*thick arrow*). The truncated Krebs cycle instead of the omitted steps of citric acid (citrate, CIT; cis-aconitate, cAC; isocitrate, ISC) is closed by transamination of OA with glutamate (GLU), which leads to formation of KGL and aspartate (ASP). As a part of the malate/aspartate shunt, these substrates enter the cytosol where OA is formed by transaminase from ASP or citrate lyase from citrate. (**B**) HIF-1α degrades in cells at normal O_2 levels after prolyl residue hydroxylation by O_2/KGL/Fe(II)-dependent hydroxylase. Hypoxia, succinate, and KGL decarboxylation by H_2O_2, which leads to decreased KGL and increased succinate, inhibits the enzyme, permitting transport of HIF-1α to the nucleus and HIF-dependent transcription of a wide variety of genes responsible for O_2 transport, vascularization, and anaerobic energy production. [Used with permission of and modified from Fedotcheva *et al.* (121)].



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